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13. ABSTRACT *(Maximum 200 Words)*

This project was aimed toward characterizing prostate cancer cell lines for responses to butyrate, and determining molecular determinants of sensitivity to butyrate-induced growth termination. Each of three prostate cancer cell lines, DU145, PC3 and LNCaP, exhibited persistent inhibition of growth after a 2 day treatment with butyrate. Most interestingly, the PC3 cell line ceased replicating but did not die, while LNCaP and DU145 underwent an initial wave of cell death. The PC3 cell could be protected from this persistent growth arrest by coculture with bone stroma, an effect also seen in a variant that exhibited enhanced growth in the absence of stroma. An additional form of butyrate response was seen in derivatives of the T24 bladder cancer cell line, which exhibit delayed onset apoptosis during butyrate treatment.

Several lines of evidence pointed to a cyclin-independent role for p21cip1 protein in the ability of prostate cancer cell lines and T24 derivatives to tolerate butyrate treatment. Most remarkably, the JCA1 variant of T24 continued to divide mitotically during butyrate treatment, arresting in the subsequent G1phase. Such arrest was however unstable, the cultures reinitiating DNA replication over the next 40 hr in association with declining level of p21cip1, increasing level of cyclin E and increasing incidence of apoptosis. A comparable phenomenon in the G2 arrest competent T24 variant, TSUPr1, suggests that butyrate-induced apoptosis was independent of cell cycle phase.

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FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. JMcB

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For the protection of human subjects, the investigators adhered to policies of applicable Federal law 45 CFR 46. JMcB

In the conduct of research involving hazardous organisms, the investigators adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Jack McBain 5 JUNE '02

PI - Signature Date

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Abbreviations

CFE	colony forming efficiency (# of colonies / # of cells plated)
HDI	histone deacetylase inhibitor
SBHA	suberoylbishydroxamate
PKC	protein kinase C activator
SDS-PAGE	SDS polyacrylamide gel electophoresis
TAU-PAGE	Triton-acid-urea polyacrylamide gel electophoresis
JCA1	T24 derivative published as being derived from primary prostate cancer
TSUPr1	T24 derivative published as being derived from primary prostate cancer
ND1	DU145 derivative published as being derived from primary prostate cancer
PC3	unique, original prostate cancer cell line from bone metastasis
DU145	unique, original prostate cancer cell line from brain metastasis
LNCaP	unique, original prostate cancer cell line from lymph node metastasis
VACO 5	colon cancer cell line
HCT116	colon cancer cell line
CKIkip	protein inhibitor of cyclin-dependent kinases (p21cip1, p27kip1, p57kip2)
CDK	cyclin-dependent kinase
TPA	protein kinase C activator
MCPA	methylenecyclopropanecarboxylic acid, an inhibitor of butyrate metabolism
H4Ac4	histone H4 with 4 acetyl substitutions

Butyrate-induced apoptosis in prostate cancer cell lines

1. Summary of goals and findings

Histone deacetylase inhibitors (HDI) such as butyrate and suberoylbishydroxamate (SBHA) have varying effects on the fate of cultured cells. Much evidence has been uncovered suggesting that many cancer-derived cells are uniquely susceptible to HDI-induced apoptosis or other forms of death, or irreversible inhibition of growth. We set out to test the hypothesis that cells with a p53-null phenotype, or more broadly with aberrant regulation of p21cip1, would be among HDI-hypersensitive cell lines. Ablation of p53 function was to be tested for its ability to sensitize HDI-tolerant cells. As an indicator of p53 protein function, the capacity for ionizing irradiation-induced expression of p21cip1 or autoinduction of p53 was tested in each of the cell lines under study. In addition, accumulation of p21cip1 and other cyclin/kinase inhibitors of the cip/kip family (CKIkip) was measured as a response to refeeding, crowding, HDI treatment and protein kinase C (PKC) activation, each considered to alter the abundance of these proteins by mechanisms distinct from those mediated by p53. Our results speak against a role for p53 protein functions in tolerance of HDI treatment, but suggest roles for the CKIkip proteins, especially p21cip1.

We have used a set of six cell lines to survey the response of prostate cancer cells to HDI treatment. It is now clear that only three of the cell lines are authentic prostate cancer derivatives (PC3, DU145 and LNCaP). The ND1 subline appears to have originated as adventitious contamination by DU145 of a primary prostate cancer culture, while JCA1 and TSUPr1 represent two similar but independent instances of adventitious contamination of primary prostate culture by cells from variants of the T24 bladder cancer line. Interestingly, the later two lines are proving uniquely informative with respect to molecular determinants of HDI tolerance, and such findings may have implications for the unique sensitivities of the three prostate cancer cell lines to HDI-induced cell death. Shared and unique molecular responses have thus been identified to aid in our search for determinants of HDI-induced growth inhibition and cell death.

Notable responses of these cell lines to HDI treatment included the following:
1) While TSUPr1 cells exhibited a typical G2 arrest during butyrate treatment, JCA1 cells continued to divide for nearly 48 hr, segregating hyperacetylated chromatin in spite of HDI-induced p21cip1 accumulation. Levels of p21cip1 and p57kip2 proteins declined spontaneously between 24 and 48 hr of butyrate treatment in cultures from TSUPr1 and JCA1, coincident with an increase in DNA replication by JCA1 and increases in cyclin E levels and apoptosis by both cell lines.
2) The ND1 cell line was more apparently more resistant to HDI-induced cell death than was DU145. Although both cell lines suffered a considerable reduction in colony forming efficiency (CFE) after butyrate treatment, dense cultures of ND1 were more tolerant than dense cultures of DU145.
3) TSUPr1 and ND1 cells appeared highly susceptible to butyrate-induced reduction in CFE when grown in standard culture medium, but such inhibition was remarkably diminished when tested in culture medium containing ascorbate and hydrocortisone, a medium used for osteoblast culture.
4) PC3 cells were highly inhibited in CFE and ability to resume logarithmic growth after butyrate treatment, but such inhibition was largely relieved when these cells were cocultured with bone-derived stromal cells. The increased CFE of such stroma-cocultured PC3 may be independent of the tolerance for butyrate, because a CFE-enhanced variant of PC3 retained sensitivity to HDI-induced reduction in CFE.
5) LNCaP cells were profoundly inhibited in cell survival after treatment with butyrate. Unlike PC3 cells, this irreversible inhibition was only modestly relieved upon coculture with bone stromal cells. The finding that basal levels of p21cip1 were maintained at a relatively high level in logarithmically

growing cultures, while only modestly increased by X-irradiation, HDI treatment or PKC activation, suggested that expression of this CKIkip protein impaired. Emerging views relating to survival-protective functions of p21cip1 suggest that such hyporesponsivity can result from deficiencies in culture conditions, especially the extracellular matrix to which the cells attach.

None of the cell lines exhibited dramatic, apoptotic HDI-hypersensitivity as was previously noted in cell lines from ovarian cancers and melanomas. A lack of either basal or HDI-induced p21cip1 accumulation has been shown to be typical of many such HDI-hypersensitive cell lines. Such hypersensitivity was apparent in certain colon cancer cell lines when a protein kinase C activator such as TPA or mezerein was included during exposure to butyrate. With the possible exception of LNCaP, the effects of treatment with a PKC activator and HDI were largely additive when measured as survival or p21cip1 protein depletion. However, the combination of the two agents led to nearly complete suppression of recovery by JCA1, ND1, LNCaP and PC3.

HDI treatment may reveal the potential of a given cell for normal deacetylase-regulated expression of inducible or cyclically expressed proteins, the later exemplified by cell cycle regulatory proteins. Such an interpretation would make sense of the nearly universal induction of cyclin E in HDI-treated cells. Changes in abundance of cyclin and CKIkip families of CDK regulatory proteins, anticipated to indicate HDI-responsive growth regulators, may account for the heterogeneity of HDI-responsiveness found among treated cell populations. While p21cip1 appears to be nearly universally derepressed by HDI treatment of normal cells, some of the most HDI-hypersensitive, cancer-derived cell lines lack HDI-induction of this protein [1, 2]. The primary mediator of contact inhibition of growth, p27kip1, is induced via HDI treatment of relatively fewer cell types, such as CaCo-2 [3] and T24 derivatives (this report), but not diploid fibroblasts [4] or DU145. The developmentally relevant p57kip2 CKIkip protein was expressed at a greater level after HDI-treatment of each of the T24 derivatives and prostate cancer cell lines (except for PC3) as had previously been reported for several cell lines from stomach cancers [5], yet was expressed at a high basal level, refractory to further HDI-induction in PC3 cells and is repressed upon HDI-treatment of Hep3B cells [6]. We believe that p57kip2 and, especially, p21cip1, but not p27kip1, may serve survival protective roles during HDI treatment.

The uniform lack of success in establishment of primary prostate cancer cell lines and the relative paucity of cell lines from metastatic prostate cancers signify the difficulties attending long term culture of prostate cancer. Of over 40 primary prostate cancers from which we attempted to derive cell lines, each culture exhibited an initial increase in epithelial and stromal cell numbers followed by a progressive decline in epithelial cell proliferation, especially after stromal cells began to die. None yielded continuous cultures despite several procedural modifications. Considering that prostate cancers are notoriously rich in stromal cell elements, and can as metastatic cancers remain limited to skeletal tissues, we set about to develop methods for coculture of fastidious prostate cancer cells using bone-derived stromal cells. These studies revealed pronounced growth enhancing effects of stromal cell culture for PC3 and LNCaP cells, and demonstrated the versatility of cytokeratin antibody discrimination of epithelial colonies for quantitative survival and growth studies. Despite such encouraging preliminary data and feasibility demonstrations, securing multiple clinical collaborators, and obtaining local peer review and IRB approvals for the trial, proposals for grant funding of this work have been uniformly rejected as non-competitive by study sections of NIH and several national cancer research charities. Such 'organotypic' simulation was recently deemed to be so trivial that supplemental funding was offered to existing NIH grant holders to develop these models in the context of pursuing popular mechanistic questions.

2. Introduction

The histone deacetylase inhibitors are a promising new class of cancer therapeutics. The prototypical HDI, butyrate salt, was shown to be a nearly universal inhibitor of growth for cultured cells, with effects that are nonetheless reversible for most cells. While many cancer-derived cell lines resemble their normal counterparts by arresting growth and expressing differentiation markers, a sizable proportion of cancer-derived cell lines instead suffer irreversible effects such as apoptosis or terminal differentiation. Early reports noted that butyrate-treated cells reversibly arrested growth with a cell cycle phase specificity similar to that seen after X-irradiation, with a large component of the cell population bearing a 4N complement of DNA consistent with G2 phase arrest. The ability to arrest in G2 (or G1) was recently suggested to correlate with survival. It is thus possible that several neoplastic mechanisms of growth enhancement fortuitously sensitize cells to HDI-induced apoptosis, although the protective role of cell cycle arrest and the nature of the trigger for apoptosis in butyrate-hypersensitive cells remain unclear.

The lack of specific understanding of the distinctions between butyrate-tolerant, -sensitive and -hypersensitive cell types has made it difficult to assess the efficacy of HDI therapy against a disease with as much intratumor and interpatient heterogeneity as prostate cancer. Normal fibroblastic cells that are either contact inhibited, i.e., in a G0 phase of the cell cycle, or logarithmically growing are highly tolerant of HDI treatment. Cells with hyperacetylated chromatin have been shown to complete DNA replication once initiated [7], subsequently arresting with a 4N complement of DNA [8, 9]. Such G2 arrest, as well as G0/G1 arrest, has typically been coincident with accumulation of p21cip1 protein [4, 10]. HDI-treated, asynchronously dividing cells therefore present with a ratio of G0/G1 and G2 phase arrested cells that reflects both the proliferative state of the cells and their capacity for phase-specific arrest. While cells arrested in both phases have been shown to be capable of survival in the presence of butyrate, cells with deficiencies in G2 phase arrest have been thought to be especially likely to undergo apoptosis [1, 2]. Furthermore, G2 arrested cells that were washed to remove butyrate were found to resume growth with a sizable proportion of replicative tetraploid cells [8, 9]. However, a final common pathway by which HDI treatment leads rapidly to cell death appears to be either elusive or nonexistent. A large proportion of ovarian cancer, melanoma and colorectal cancer cell lines respond within 24-48 hr HDI treatment with high levels of apoptosis [1, 2, 11, 12], but claims as to primary determinants of HDI-hypersensitivity have largely given way to numerous exceptions.

The CKIkip proteins are three unique but structurally related proteins that share an ability to inhibit cyclin-dependent kinases (CDKs), and bind both cyclins and CDK proteins [13]. The prototype of this family, p21cip1, was identified as a mediator of p53 protein-dependent growth arrest as a response to DNA damage. Subsequent investigations have identified p27kip1 as a mediator of contact inhibition of cell growth, and p57kip2 as a CDK inhibitor espressed prominently by postreplicative and terminally differentiated (but living) cells. As a result of continued investigation, the p21cip1 protein has also been found to have a potential role in differentiation, senescence and stress responses independent of p53 involvement.

The ability of p21cip1 to catalyze the assembly and activation of cyclin/kinase complexes [14], and the inhibitory actions of p21cip1 on caspase 3 activation [15] are examples of growth stimulatory and apoptosis-inhibitory actions seen at low and high abundances of p21cip1. It is thus no longer considered paradoxical that p21cip1 deletion is rare among human cancers or that aggressive carcinomas can express high basal levels of p21cip1. The finding of cytoplasmic p21cip1 in association with ASK kinase [16] is similarly associated with enhanced survival. However, common coprecipitation and immunodepletion studies have indicated that quantitatively the major binding partners of CKIkip proteins in normal cells are cyclin/CDK complexes themselves, with other moderate affinity ligands coming into prominence only at high levels of induction. It is possible that similar considerations apply to p27kip1 and p57kip2. Similarly, posttranscriptional mechanisms of protein expression, which play the major role for regulating

p27kip1 levels, may modify p21cip1 persistence. For example, in an example of what may prove to be a positive feedback cycle for apoptosis resistance and induction, p21cip1 is both an inhibitor and substrate caspase 3 activity. The CKIkip proteins may thus be prime factors for tonic control of major decisions in both cell growth and cell survival.

An association of low inducibility of p21cip1 has been demonstrated for many of these cancer-derived, HDI hypersensitive cell lines, further evidence for a possible specificity of HDI-induced apoptosis for cells with altered replication checkpoint functions. Recent studies have however shown that HDI-hypersensitive cells, incompetent for HDI-induced p21cip1 expression and G2 arrest, will nevertheless die if HDI treated while blocked in cycle progression prior to or during DNA replication [2]. Despite evidence pointing to the existence of separable G1/S and G2/M 'checkpoints' that each correlate with p21cip1 expression [17, 18], it has not been possible to determine whether G2 arrest is essential for survival in p21cip1 expression-competent HDI-treated cells. Such a result would support the hypothesis that p21cip1 can act as a survival factor irregardless of cell cycle phase. Rather, contradictory findings are available, such as the demonstration that fibroblasts from p21cip1-null mice nevertheless arrest at the G1/S transition and presumably survive during HDI treatment [19]. While associated with HDI-induced reduction in cyclin D levels, questions remain as to whether the survival of such HDI-treated cells reflects the actions of, e.g., HDI-induced p57kip2.

A paradoxical phenomenon has come to light, in which moderate basal expression and low magnitude of inducibility of CKIkip proteins is effected as a general consequence of adverse culture conditions. High basal and low radiation-inducible expression of p21cip1 by fibroblasts normalizes when cells are attached to a those extracellular matrices associated with the improved survival of irradiated fibroblasts plated on collagen or fibronectin rather than culture plastic [20, 21]. We hypothesize (or predict, given the absence of resources to ask the question) that the altered responsiveness to HDI inhibitors that we see upon coculture of PC3 cells with stroma will be attributable to such context-specific CKIkip expressibility. This provides another argument to justify development of better culture environments for growth of prostate cancer cells.

Prostate cancers at all stages of neoplastic progression have relatively low efficiencies of survival as isolated epithelial cells. While this is particularly true of treatment-naive, primary prostate cancers, reports of successful establishment of cell lines from metastatic tumor are also few. Even among xenografts, reports of successful establishment mention use of whole tissue explants (stroma plus epithelial glands) and mention little about the long term stability of serial transplants. Because current prostate cancer cell lines are so few, we speculate that they represent either unusual cases of prostate cancer, or selection *in vitro* of cellular variants with unusually autonomous growth. Against this background, our studies of differential responsiveness of the 3 prostate cancer cell lines to HDI treatment may reveal alternative strategies for autonomous survival of individual cell lines rather than a response typical of prostate cancers as a group. More importantly, we found that HDI-responsiveness by each of the three prostate cancer cell lines was uniquely affected by coculture with bone-derived stroma. Conventional cell culture studies of available prostate cancer cell lines may thus have limited applicability to prostate cancer cells as they exist in tumors, where interactions with stroma may be not only essential, but decisive in treatment outcome following challenge with growth inhibitors.

3. Original Aims

a. Assess the timing of growth arrest and recovery, and the surviving fraction for cultured prostate cancer cell lines treated with histone deacetylase inhibitors (HDI). A major subaim was therefore to attempt to derive cell lines from primary prostate cancers. It was assumed that a subset of these tumors, corresponding to that percentage of prostatectomized men who suffer recurrence of metastatic prostate cancer, would contain clonogenic cancer cells.

b. Determine the relationship of p53-system functional integrity to HDI tolerance, hypothesizing that cells lacking p53 functions would be most susceptible to butyrate-induced apoptosis. Butyrate-resistant prostate cancer cell lines, predicted to be those with wild-type p53 functions, were to be genetically altered, by transfectional expression of the papillomaviral oncogene E6. According to the hypothesis, ablation of major p53 transcriptional functions would sensitize prostate cancer cells to butyrate-induced apoptosis.

Revised statement of work - It was rapidly apparent that Xray induction of p21cip1 and p53 autoinduction had little correlation with HDI tolerance among 'prostate cancer' cell lines. Furthermore, it was becoming clear that differences in HDI-induced growth arrest between JCA1 and the other lines, and among the fastidious cell lines (later shown to be *bona fide* prostate cancer lines) could be related to peculiarities of CKIkip protein expression. We therefore chose to investigate differences among HDI-induced cyclins and CKIkip proteins that might explain HDI-intolerance by cells such as LNCaP, and resistance to HDI-induced growth arrest in JCA1. In the end, it may be that Xray-responsiveness of p21cip1 expression by JCA1 will shed light on the possible survival advantage of certain unusual p53 mutations [22].

year one	growth rate and survival assessments of the 6 'prostate' cancer cell lines
	comparison of responses to butyrate, mezerein and the combination
	preliminary determination of apoptosis incidence and timing
	measurement of potential indicators of differentiation state
	initial determination of Xray response, re: p53 and p21cip1 expression
	43 primary prostate cancer cultures intended to attempt cell line establishment
year two	butyrate and SBHA timing and dose response
	preliminary development of stromal / cancer coculture and evaluation methods
	cyclin, CKI expression - timing and comparison of HDI and PKC activator effects
	preliminary cytofluorography assessments of cell cycle perturbations
year three	HDI and PKC activator response in stroma coculture
	cytofluorography for timing, quantitation and correlation with survival
	DNA replication determinations in T24, JCA1 and TSUPr1 clonal sublines
ongoing	substrate dependence of basal and HDI-induced CKIkip protein expression

4. Ultimate Aims, Results and Hypotheses

a. Response of 'prostate cancer' cell lines to butyrate treatment

Studies on cultures of human cancer cell lines and their transplantable xenograft counterparts suggest that the potential of HDI agents for prostate cancer treatment may be better than for any other malignant disease category. As an illustration, the regression of the CWR22 xenograft line upon treatment with hydroxamate HDI's [23] was effected at doses less than would have been needed to continuously

maintain hyperacetylated histones. The later paper also presented results indicating a high level of cell death in acylhydroxamate-treated LNCaP cultures that was not seen in PC3 and TSUPr1 cultures, despite a comparable level of cell growth inhibition. While these investigators did not examine recoverability of logarithmic growth rates by PC3, the earlier study by Halgunset [24] found that such recovery was heterogeneous and that other phenotypic consequences (such as morphological change and fibronectin expression) indicated a complex responsiveness.

Serial hemacytometer counts ('growth curves' - figs 1a-d) and colony formation assays (tables 1 and 2) of cells from cultures treated for 24-72 hr with butyrate were examined to compare the rapidity of growth arrest and loss of cell numbers with the ultimate change in the surviving fraction. The cell lines DU145 and LNCaP were found by multiple measures to be sensitive to HDI-induced death, while PC3 was simply found to have become more dependent upon stromal or autocrine factors after treatment. The ND1, JCA1 and TSUPr1 cell lines were at first considered to be relatively butyrate-tolerant, i.e., little or no initial cell loss, and rapid recovery of growth after removal of butyrate (figure 1). In fact, by some measures TSUPr1 appears to be very sensitive to butyrate-induced cell death (tables 1 and 2). The JCA1 cell line on the other hand exhibited an initial increase in cell numbers during butyrate treatment, but with continued treatment suffered considerable cell loss (figures 1c and d). None of the cell lines could be said to be hypersensitive in a manner similar to the colon cancer cell lines we have examined [11], or as observed among melanoma and ovarian cancer cell lines studied by Gabrielli, et. al. [1, 2] and Langdon, et. al. [25].

Table 1 Colony forming efficiency of prostate cancer cell lines and T24 derivatives effect of 48 hr butyrate treatment.

cell line	date and medium	control CFE	butyrate CFE
ND1	17 Oct. '00 - D8F	0.197+/-.006	0.002+/-.002
ND1	3 Jan. '01 - Skelgro	0.240+/-.012	0.094+/-.010
DU145	2 July '99 - D8F	0.190+/-.014	<0.001
DU145	30 Oct. '00 - Skelgro	0.130+/-.014	0.006+/-.001
PC3	2 July '99 - D8F	0.014+/-.003	<0.001
PC3	24 Oct. '00 - Skelgro	0.07+/-.02	0.001+/-.001
LNCaP	27 Oct. '00 - Skelgro	0.050+/-.006	<0.001
JCA1	13 July '99 - D8F	0.54+/-.04	0.28+/-.02
JCA1	23 Mar. '01 - Skelgro	0.34+/-.05	0.22+/-.03
TSUPr1	13 Nov. '00 - D8F	0.51+/-.07	0.017+/-.007
TSUPr1	15 Nov. '00 - Skelgro	0.52+/-.01	0.35+/-.02

Cells were removed from the dish and disaggregated using trypsin/EDTA. After sieving through 30 μm nylon mesh, cells were plated on cell culture plastic at between 200 and 1000 cells per 10 cm^2 dish in either standard culture medium (DMEM with 8% fetal bovine serum - D8F) or D8F with 100 μM ascorbic acid 2-phosphate and 1 μM hydrocortisone hemisuccinate (Skelgro). After 2-3 days incubation, butyrate or control medium was added as a 4X concentrated solution. After a 48 hr treatment, media was removed with washing and recovery of non-adherent cells, which were returned to the dish in fresh medium. After an additional 2-3 week growth period, cell colonies were dried, fixed with ethanol and stained with methylene blue. Colonies of ≥30 cells were counted using a microprojector. Data are the colony forming efficiency (# of colonies / # of cells plated) +/- standard deviation.

Figure 1. Growth rate determinations for butyrate-treated cell lines.

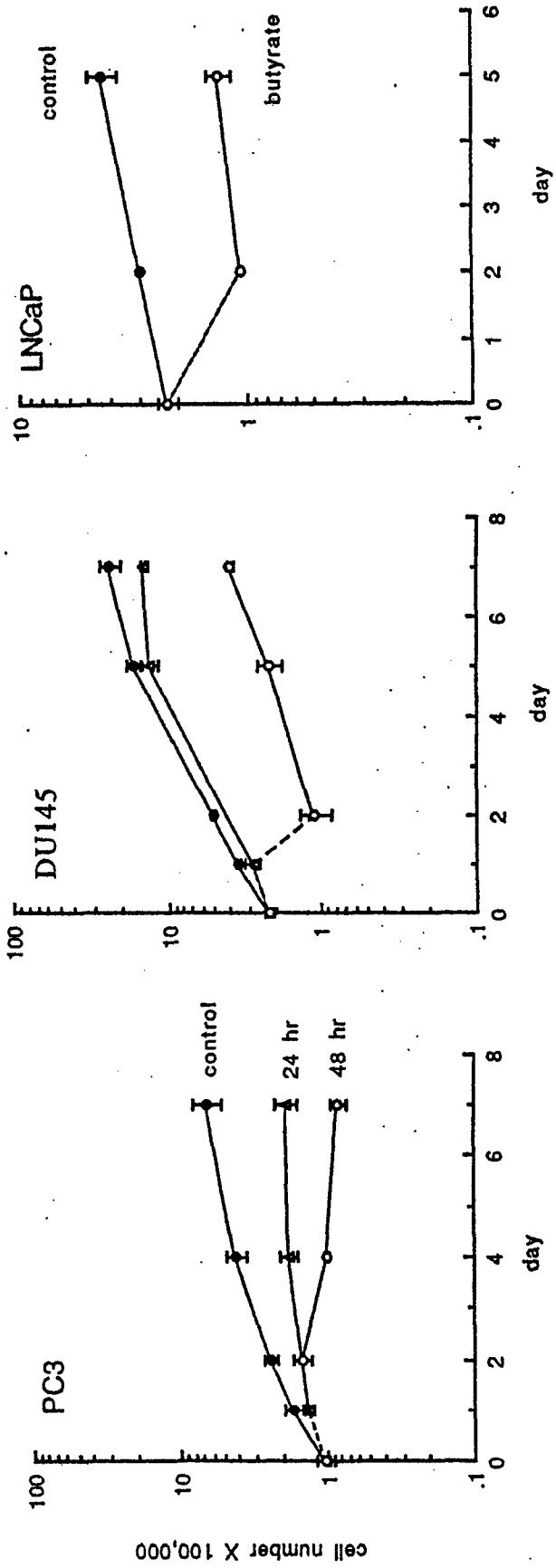
Cells were disaggregated and seeded into replicate dishes at 1-2 X 10^5 cells per 10 cm^2 dish and incubated for 2-3 days before beginning treatment. Butyrate was added as 1/3 volume of a 4X concentrate, and time zero counts were performed. After 2 or the indicated times of treatment, media was removed and the non-adherent cells sedimented, washed and added back to the dish in fresh medium. After a further incubation, cells were collected by centrifugation of medium and trypsinization of adherent cells with trypsin/EGTA/dextran sulfate 8000. Viable cells were counted with a hemacytometer, using trypan blue to stain non-viable cells. Data are the average of three replicate determinations +/- standard deviation.

figure 1a. Growth rate of prostate cancer cell lines.
24 or 48 hr treatment with butyrate (5 mM).

figure 1b. Growth rate of T24 (JCA1 and TSUPr1) and DU145 derivatives.
48 hr of 5 mM butyrate treatment.

figure 1c. JCA1 growth and death rates.
Continuous 5 day treatment with n-butyrate or isobutyrate.

figure 1d. Effects of continuous butyrate on JCA1 and TSUPr1 growth/death.



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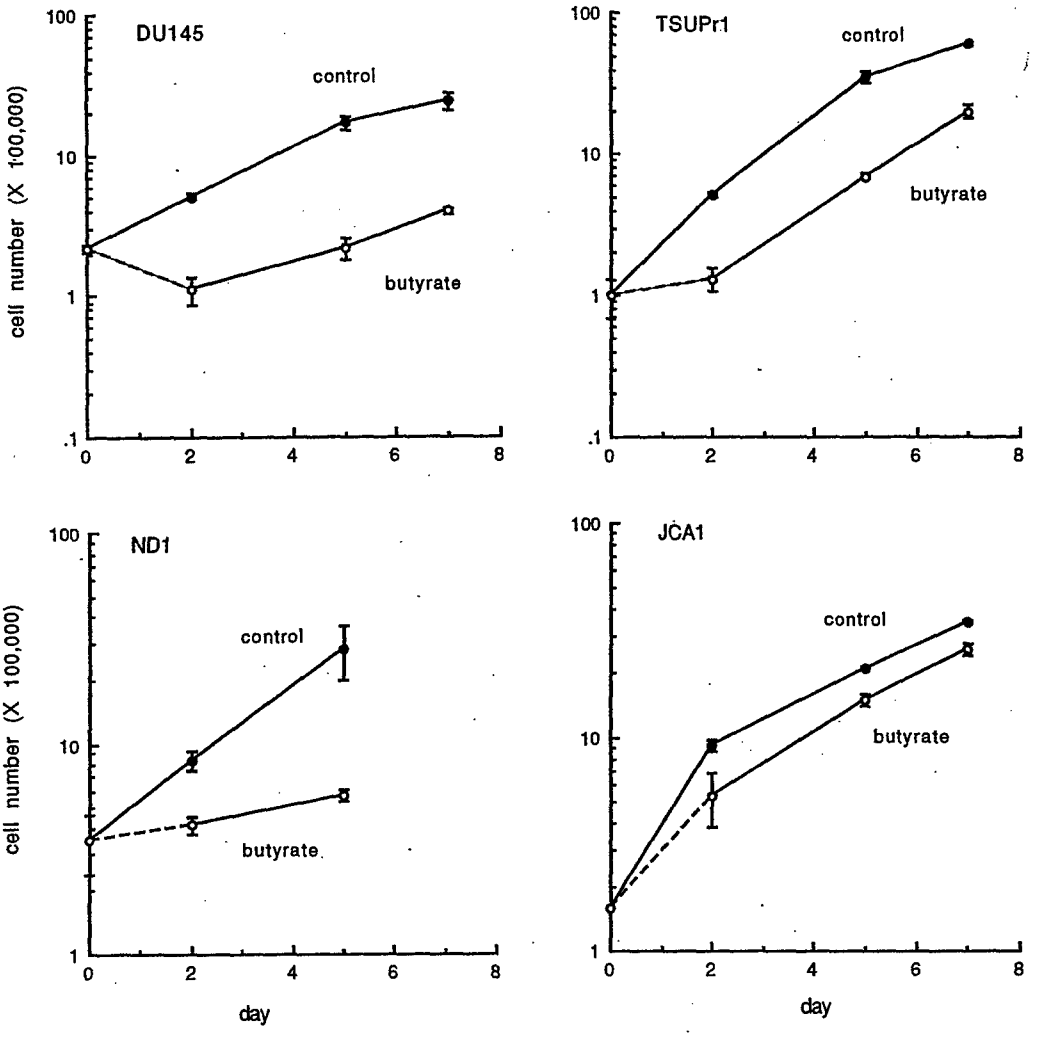


figure 1b. Growth rate of T24 (JCA1 and TSUPr1) and DU145 derivatives. 48 hr of 5 mM butyrate treatment.

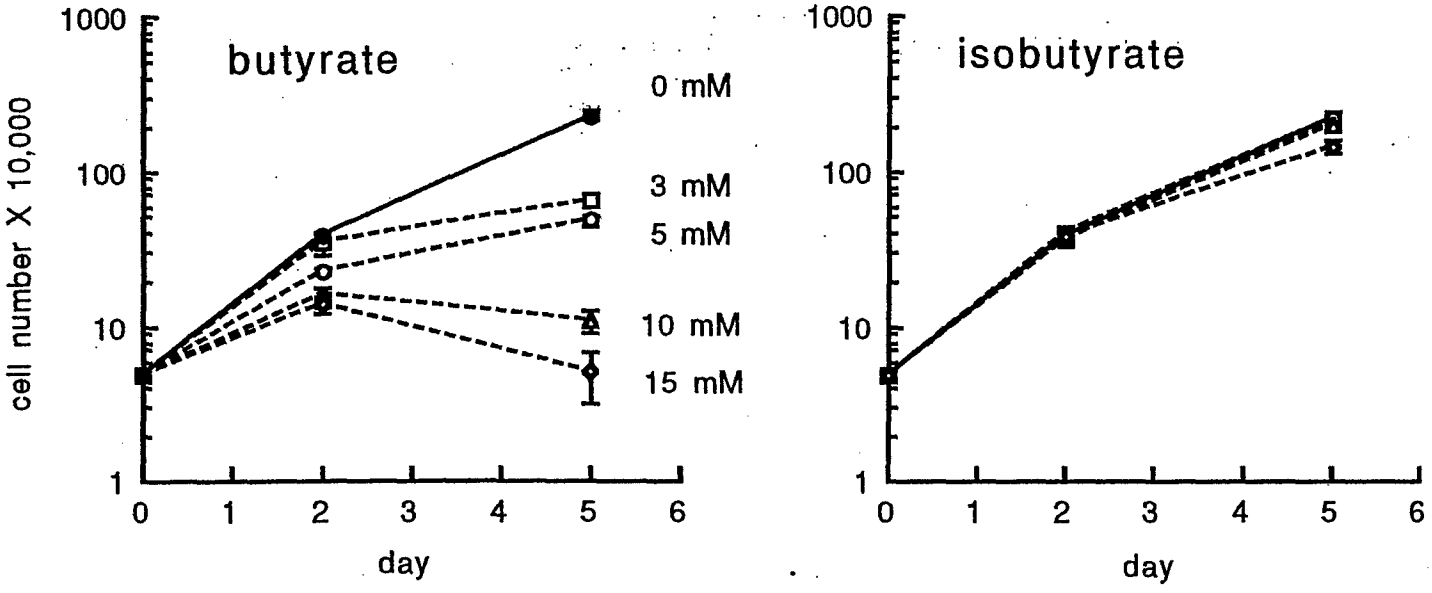


figure 1c. JCA1 growth and death rates.
Continuous 5 day treatment with n-butyrate or isobutyrate.

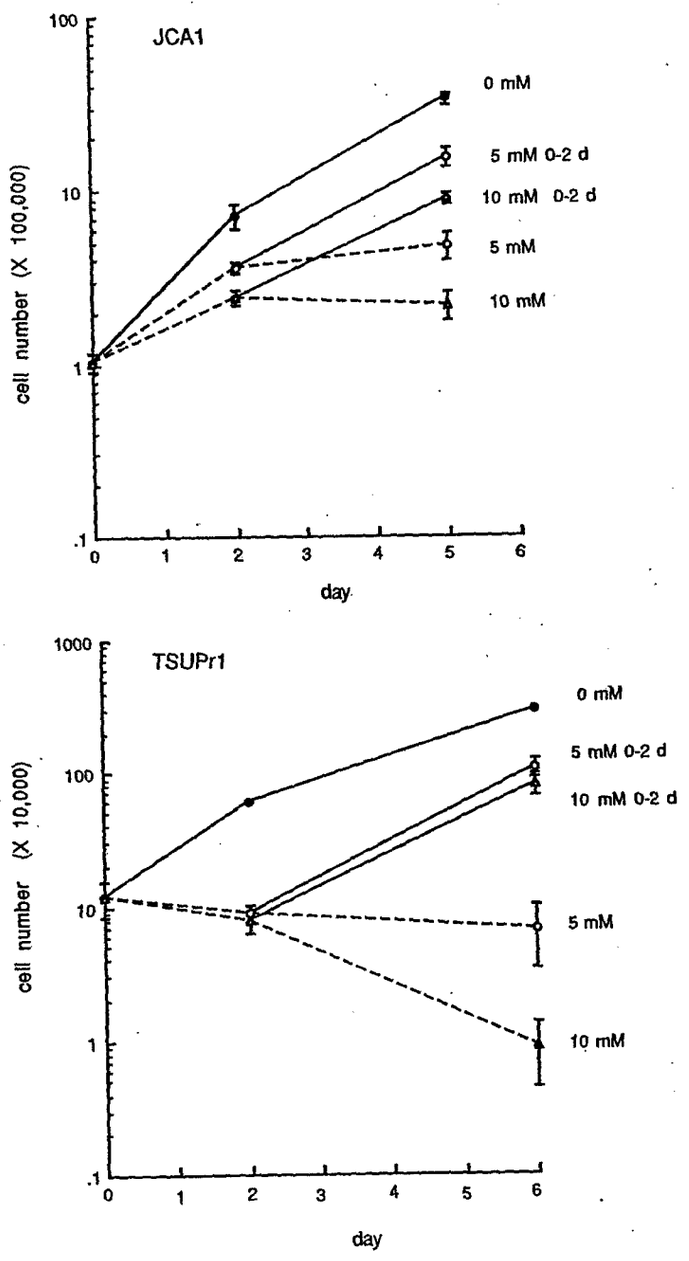


figure 1d. Effects of continuous butyrate on JCA1 and TSUPr1 growth/death.

Table two Effect of butyrate treatment time on TSUPr1 CFE

time of treatment	colony forming efficiency
control	0.412+/-.039
24 hr butyrate	0.099+/-.006
48 hr butyrate	0.029+/-.003
72 hr butyrate	0.01+/-.003

TSUPr1 cells were disaggregated and plated at 1000 cells per well in DMEM 8%FBS. After two days, butyrate treatment (5 mM) was initiated, and after periods of 24, 48 and 72 hr treatment was terminated by washing and refeeding. After two weeks for colony formation, dishes were dried, fixed with ethanol and stained with methylene blue. Colonies of >30 cells were counted using a microprojector. Data represent the average of three dishes +/- standard deviation.

Our findings of a net reduction in cell numbers by HDI-treated LNCaP and DU145 cell cultures (figure 1a) and of large magnitude loss of colony forming ability (CFE) after 48 hr of butyrate treatment of several cell lines (table 1), and the greater tolerance of mass cultured TSUPr1 (figures 1b and d) are in line with these published findings. However, our additional observations reveal elements of HDI response requiring further definition of tolerance and sensitivity to HDI-induced cell death. For example, when cultures were tested at low seeding densities in standard growth medium, each of the cell lines except JCA1 was severely inhibited in colony formation after 48 hr of butyrate treatment (table 1). However, when we determined CFE in osteoblast growth medium, essentially standard growth medium supplemented with ascorbate and hydrocortisone, CFE of butyrate-treated cultures of TSUPr1 and ND1 was dramatically increased. We have not yet pursued this phenomenon.

The case of PC3 cell response is particularly intriguing, as it became clear that persistent growth inhibition seen on cell number growth curves (figure 1a) was not death and indeed was not necessarily persistent. The clear nonlinearity of CFE with input PC3 cell number, ranging from 0.4% to 40% depending on seeding density or use of feeder cells, meant that no single assay could be used to describe the survival of HDI-treated PC3 cells. Considerably greater linearity was evident when we used bone stromal cell cultures as a 'feeder' system to meet the residual requirement of this 'autonomous' prostate cancer cell line for stromal products. As presented later in this report, the actions of stromal cells upon PC3 would be sufficient to change the designation of PC3 from an HDI-sensitive to an HDI-tolerant cell line.

We assumed that we would also need to measure death by several criteria. We found that our estimate cumulative apoptosis during treatment, using a measure of apoptotic proteolysis, correlated well morphological observations and with the more non-specific determination of subG1 cells by flow cytometry. However, neither corresponded to the losses in CFE or cell numbers that accompanied butyrate treatment of LNCaP, DU145 or PC3. Furthermore, the apparently HDI-tolerant JCA1 cell line exhibited considerably more apoptotic protein cleavage (figure 2a) and subG1 cells (figure 3a) than did the DU145 cell line, which suffered considerable cell loss during 48 hr of butyrate treatment (see figure 1a). In both TSUPr1 and JCA1 cultures, the appearance of apoptotic (subG1) cells that was evident by either flow cytometry, or assay of apoptotic protein fragmentation appeared to correlate temporally with CFE losses.

Our subline of DU145 cells, obtained from ATCC, exhibited an approximately 15% maximal extents of apoptotic proteolysis (figure 2a) and low yields of sugG1 cells while suffering >50% loss in cell number and reduction in CFE after 48 hr butyrate treatment. In collaboration with SAIC Frederick, we have characterized the butyrate responsiveness of several of the cell lines in the 60 cell line NCI panel that is

Figure 2. Apoptotic proteolysis as an estimate of cumulative apoptosis.

Cells were treated as growing cultures (typically 50% of maximum surface coverage), by adding 1/3 volume of 4X concentrated mezerein (final concentration 100 nM), butyrate (final concentration 5 mM) or control medium (0.025% final [ethanol]). After the indicated time of treatment, detached cells were collected from the culture medium by centrifugation, while adherent cells were lysed in place with SDS, N-ethylmaleimide, EDTA and other protease inhibitors in a tris-buffered saline. The combined lysates were stored at -20°C and assayed for total protein (BCA). Aliquots corresponding to equal protein (50-100 µg/ml), reduced with mercaptoethanol and boiled, were electrophoretically separated using a Laemmli discontinuous buffer in an SDS polyacrylamide gel. Separated proteins were electrophoretically transfered from the gel to PVDF membranes. After blocking non-specific surfaces of the membranes using skim milk and BSA, specific proteins and their cleavage products were localized using monoclonal antibodies to protein kinase C epsilon (PKCe) or polyADPribosyl-polymerase (PARP), and peroxidase-conjugated antibodies to mouse or rabbit immunoglobulin, respectively. Immunoperoxidase was detected and recorded on Xray film using ECL luminol reagent (Amersham). Darkened areas on these films were semiquantitated using a transmission scanner and computer to collect digital tiff files for analysis by the NIH Image 1.61 program. None of the images were saturated for more than 5% of the analyzed bands. Backgrounds were subtracted as manual straightline estimates corresponding to local averages. Values plotted in these graphs correspond to the relative optical density of peaks corresponding to cleavage fragments (45 and 38 kDa for PKCe and 85 kDa for PARP) relative to the total density of these fragments plus holoprotein (95 kDa for PKCe and 115 kDa for PARP).

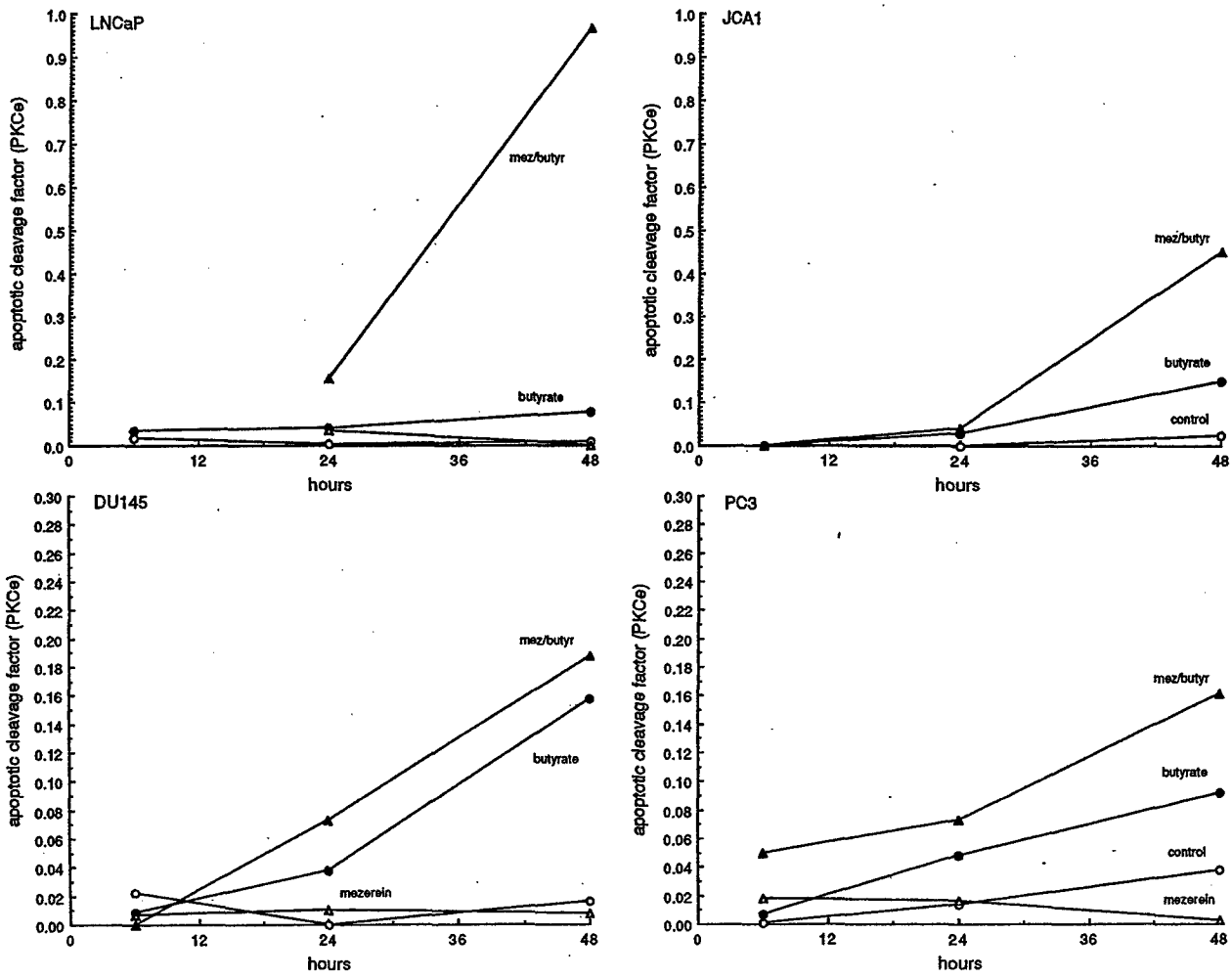


figure 2a. Comparison of LNCaP, JCA1, DU145(ATCC) and PC3 cultures for apoptotic proteolysis of PKCepsilon.

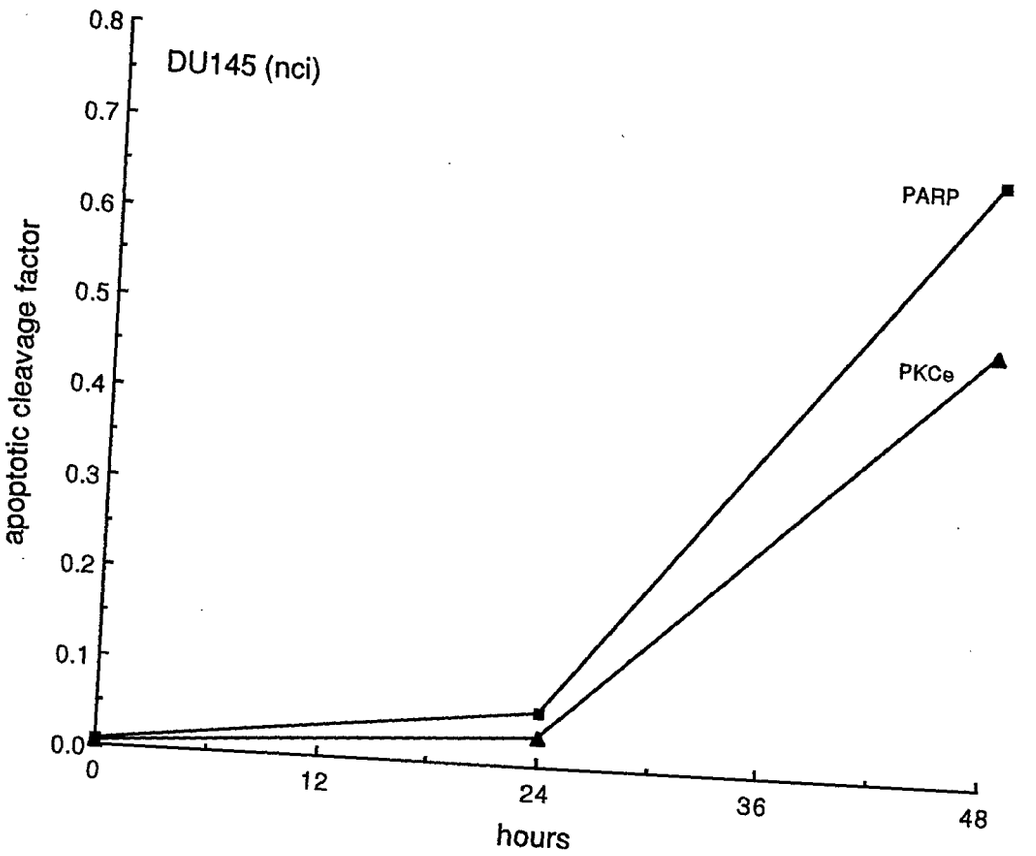


figure 2b. Butyrate treatment (5 mM) effects on apoptotic proteolysis by NCI strain of DU145.

Figure 3. Flow cytometric analysis of cell cycle arrest and apoptotic DNA loss.

Cells were treated as sparse growing cultures (typically 30-50% of maximum surface coverage), by adding 1/3 volume of 4X concentrated butyrate (final concentration 5 mM) or control medium. After the indicated time of treatment, detached cells were collected from the culture medium by centrifugation, while adherent cells were removed with trypsin/EDTA. After washing once in phosphate-buffered saline, cells were fixed in 70% ethanol before storing at -20°C. Prior to analysis, cells were sedimented, resuspended in PBS and labeled with propidium iodide in RNase A-containing PBS at 36°C. Labeled cell suspensions were sieved through 30 µm nylon mesh and analysed on a FACScan flow cytometer running CellQuest software.

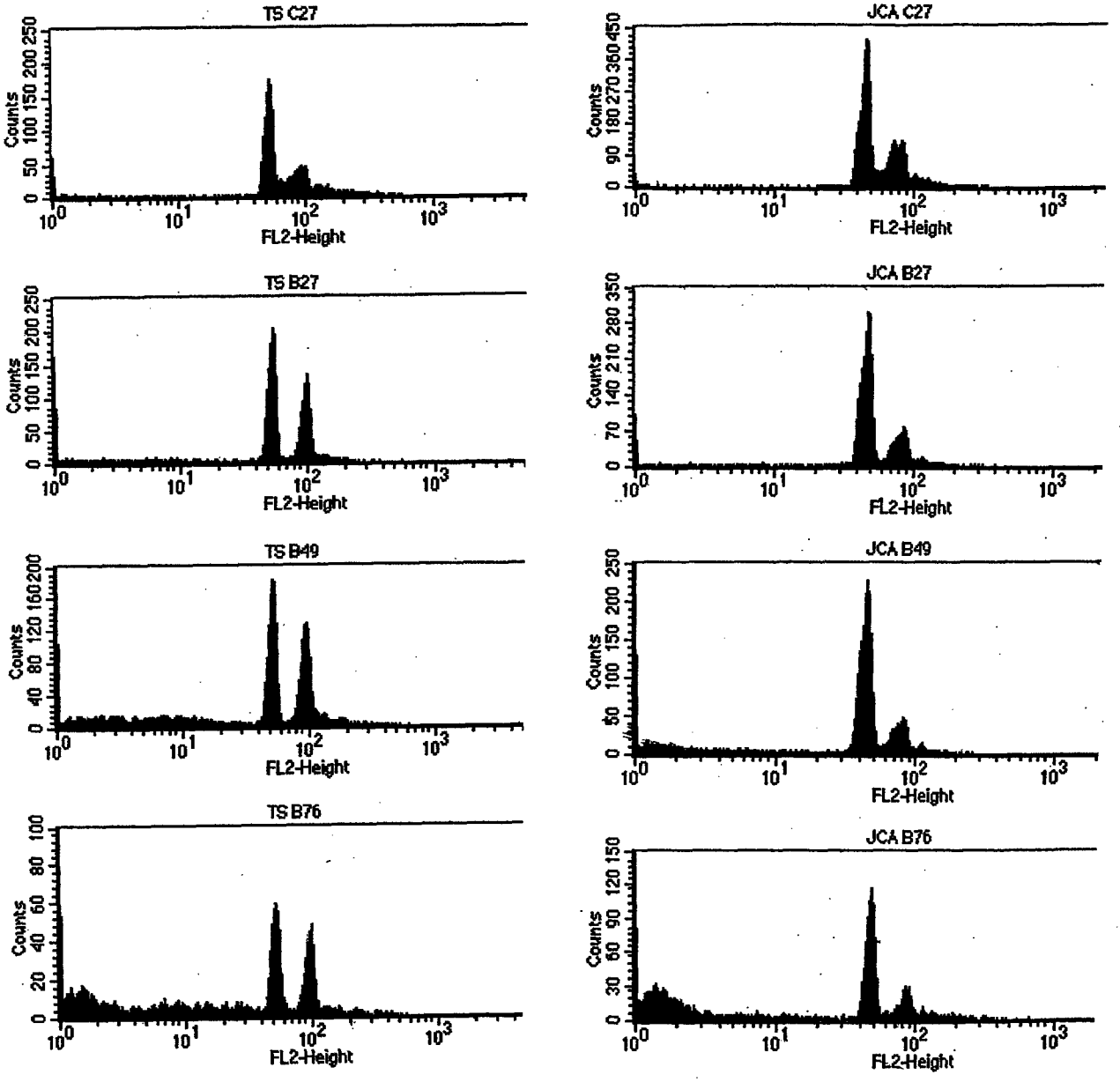


figure 3a. JCA1 and TSUPr1 PI-DNA profiles - 24-72 hr of butyrate treatment

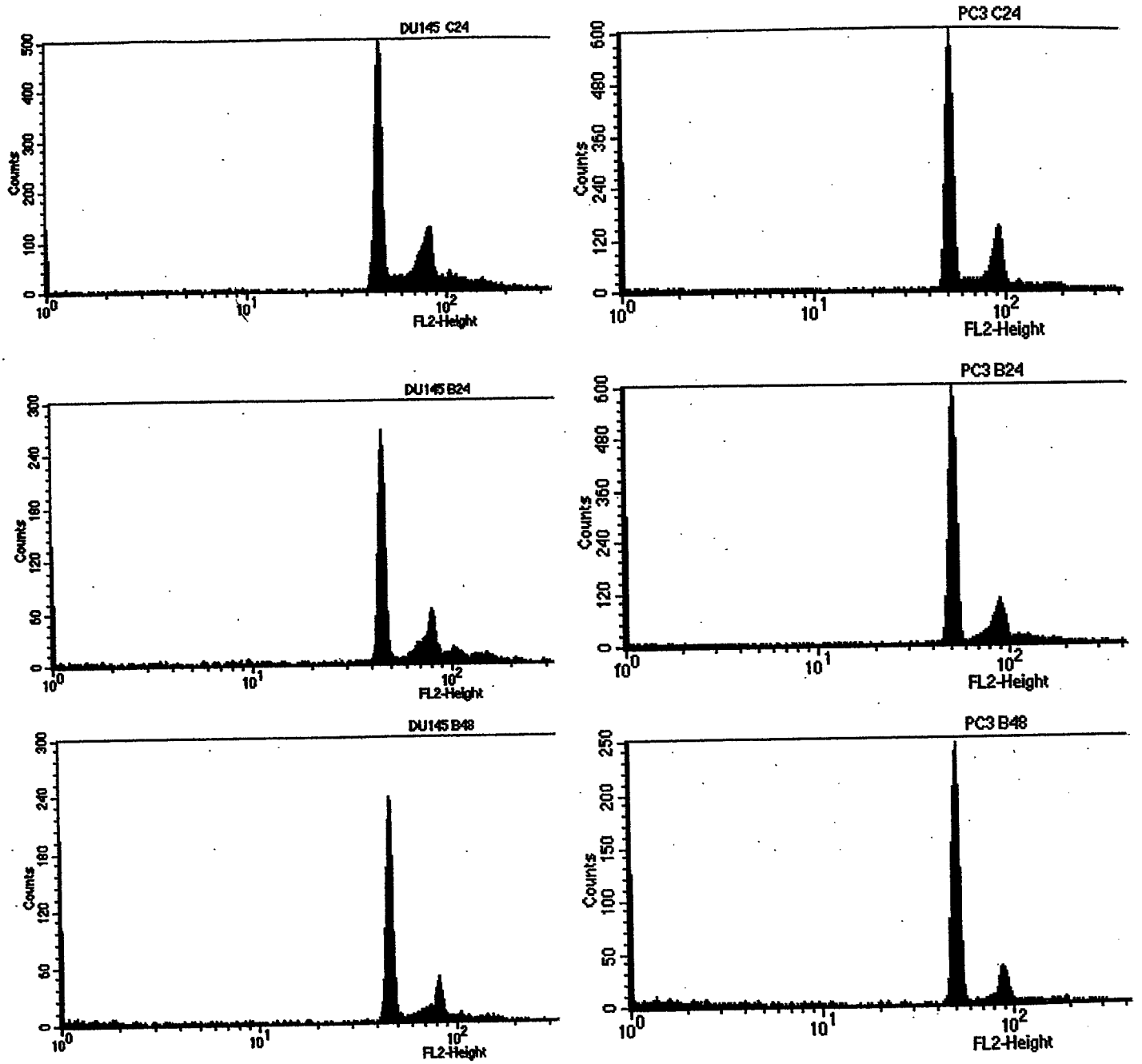


figure 3b. DU145 and PC3 PI-DNA profiles - 24-48 hr of butyrate treatment

used for comparative drug sensitivity testing. Lysates from the NCI strains of the PC3 and DU145 cell lines were treated for 24 or 48 hr with butyrate. Estimates of apoptotic proteolysis in PC3 cultures yielded approx. 10% of maximal apoptotically fragmented PARP and PKC epsilon, agreeing with our findings. Their cultures of DU145, however, yielded 50-70% maximal cleavage of PKCe and PARP, respectively (figure 2b). We have not yet obtained a seed stock of these cells to determine whether they would in our hands prove less susceptible to apoptosis. However, our finding of >50% loss in cell numbers suggests that the finding of apoptosis may be less important to our estimation of butyrate tolerance, and that our DU145 cells (obtained from ATCC) may nonetheless have died with 2/3 of the cells either failing to go through such apoptosis or proving unrecoverable.

b. Relationship of p53 expression to survival after butyrate treatment.

Our earlier work with colorectal cancer had suggested that p53 wild type cells might on average tolerate HDI treatment better than p53 null cell lines. We had seen that normal fibroblasts and the HCT116 colon cancer cell line (p53wt/p21wt) were fully resistant to butyrate-induced apoptosis despite growth arrest, while the HDI-induced apoptosis-susceptible cell lines VACO5 and COLO201 were deficient in p53 functions.

The finding of differences in survival of prostate cancer cell lines after butyrate treatment gave us an opportunity to assess the hypothesis that p53 function would correlate with survival of HDI-treated cells. The results would also facilitate selection of an experimental subject for genetic manipulation. We therefore tested each line for expression of p21cip1 and p53 proteins after exposure to a moderate dose of ionizing radiation using an ^{137}Cs gamma source and harvesting total cell proteins 24 hr after irradiation. Immunoblot analysis was used for comparison of expression of p21cip1 and p53 protein abundance (figure 4). It was expected that cells with absent or non-functional p53 protein would be deficient in X irradiation-induced expression of these proteins, while cells with intact p53 functions would accumulate increased amounts of each. The results showed that the PC3 cell line expressed insignificant amounts of p53 protein, and after irradiation little or no increase in p21cip1, consistent with a p53null phenotype. The DU145 and ND1 cell lines expressed abundant p53 protein both before and after irradiation but exhibited very low levels of p21cip1 protein after 24 hr incubation following exposure to a range of radiation doses. The TSUPr1 cell line expressed a moderate amount of a protein at 53 kDa, but neither this nor the complement of p21cip1 protein changed after irradiation. The LNCaP and JCA1 cell lines, and bone stromal cells (a p53wildtype control) on the other hand accumulated p21cip1 protein for 24 hr after irradiation. LNCaP and bone stromal cells exhibited substantial basal expression of p21cip1 relative to the other cell lines examined, and densitometrically measureable activity increased by just 20% after exposure to up to 800 cGy. JCA1 exhibited a low basal level of p21cip1, and at 24 hr after irradiation had about 3 times the control cell level (figure 4a). Autoinduction of p53 protein expression was evident after irradiation of JCA1, LNCaP and bone stromal cells, as presented in figure 4b. By both measures, therefore, radiation-responsive transcriptional stimulation of p53 target genes was evident in each of these three cell lines. Preliminary experiments indicated that treatment (-24 hr) of JCA1 with camptothecin, a topoisomerase 1 inhibitor that generates DNA strand breaks, also resulted in an approximately two fold increase of p21cip1 protein accumulation, while camptothecin treatment of TSUPr1 had no apparent effect on the abundance of this protein.

Recent work has shown that the TSUPr1 and JCA1 cell lines are derived from the T24 bladder cancer cell line [26]. These investigators sequenced what they considered to be the sole remaining allele of the p53 gene in each cell line and found identical point mutations, specifically at codon 126, TAC -> TAG (Tyr -> stop). These investigators also claimed that no immunohistochemically recognizable activity was evident in these cells. Our results with immunoblotting clearly identify a protein that comigrates with a 53 kDa protein that is expressed modestly in bone stromal cells and abundantly in DU145 and ND1.

Figure 4. Ionizing radiation responsiveness in regard to p21cip1 and p53 induction.

Cells were irradiated as growing cultures using a ^{137}Cs gammator, varying time at a constant dose rate. Cells were reincubated and harvested after 24 hr. Detached cells were collected from the culture medium by centrifugation, while adherent cells were lysed in place with SDS, N-ethylmaleimide, EDTA and other protease inhibitors in a tris-buffered saline. The combined lysates were stored at -20°C and assayed for total protein (BCA). Aliquots corresponding to equal protein (50-100 µg/ml), reduced with mercaptoethanol and boiled, were electrophoretically separated using a Laemmli discontinuous buffer in an SDS polyacrylamide gel. Separated proteins were electrophoretically transfered from the gel to PVDF membranes. After blocking non-specific surfaces of the membranes using skim milk and BSA, specific proteins and their cleavage products were localized using monoclonal antibodies to p21cip1 or p53, and peroxidase-conjugated antibodies to mouse or rabbit immunoglobulin, respectively. Immunoperoxidase was detected and recorded on Xray film using ECL luminol reagent (Amersham). Darkened areas on these films were semiquantitated using a transmission scanner and computer to collect digital tiff files for analysis by the NIH Image 1.61 program. None of the images were saturated for more than 5% of the analyzed bands. Backgrounds were subtracted as manual straightline estimates corresponding to local averages. Values plotted in these graphs for p21cip1 correspond to the relative optical density of peaks corresponding to total activity in the p20 area (sometimes p21cip1 protein was spread over a wide area apparently corresponding to the difference between the SDS front (near 18 kDa) and the salt front.

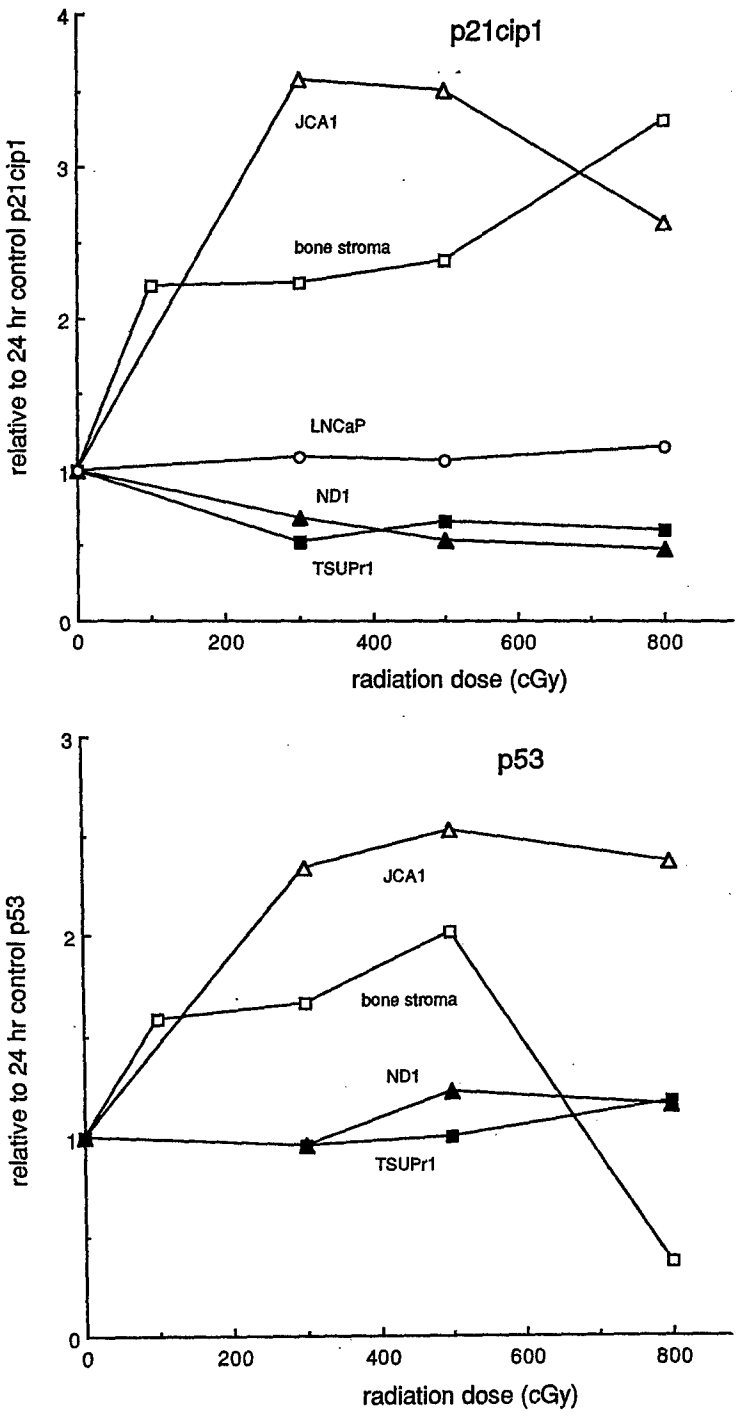


figure 4a. p21cip1 expression after irradiation of cell line cultures

figure 4b p53 protein expression after irradiation of cell line cultures

Moderate abundance 53 kDa proteins in JCA1 and TSUPr1 were previously reported by Rokhlin et. al. .[27]. Previous work indicated that all tested sublines of the T24 cell line carry an allele of the p53 gene that has suffered an interstitial deletion of three base pairs, while one subline also carries an allele with the Y126stop point mutation described above [22, 28]. Interestingly, the interstitial deletion yielded a moderately expressed protein that is missing Y126 and that failed to bind the SV40 T Ag protein [28]. Interestingly, this later property would have likely rendered futile our attempts to block p53 functions with papillomavirus E6 expression vectors. The T24 cell line is commonly thought to be null for p53 transcriptional stimulatory actions, although we haven't yet tested our T24 (ATCC) line for either the presence of immunodetectable proteins nor for radiation responsiveness of p21cip1-expression. It remains problematic that we have Xray-responsive transcriptional activity specific to JCA1, in that we remain in no financial position to deal with reviewers who might demand molecular or cellular characterization of these phenomena. However, we remain excited that such residual p53 activity might be related to other phenotypic features unique to this cell line, such as its ability to survive long term starvation and to become contact inhibited in its growth.

c. Pairwise comparisons of HDI responsiveness among 'prostate cancer' cell lines

Comparison of SBHA and butyrate as inducers of growth arrest and cell death

One possibility that was originally entertained for the continued growth of JCA1 in the presence of butyrate was a deficiency in accumulation of hyperacetylated core histones, as noted in many cell lines selected for resistance to HDI-induced growth inhibition. Thus JCA1 and DU145 were treated for various lengths of time with butyrate and SBHA, an acylbishydroxamate HDI. Total chromatin was isolated from cells, and then treated with protamine and urea to elute bulk histones. Histone species were electrophoretically separated on Triton acid urea gels and stained with Coomasie blue, as shown in figure 5a. The effect of HDI treatment was distinct for JCA1 and DU145. The later cell line exhibited patterns similar to the H4Ac4-predominant pattern that we had seen in the butyrate-tolerant HCT116 cell line, while JCA1 expressed a pattern with nearly equivalent levels of H4Ac2, H4Ac3 and H4Ac4 (and H3Ac2 & H3Ac3), a pattern reminiscent of the butyrate-sensitive VACO 5 cell line. Each of the two HDI agents caused accumulation of hyperacetylated (lower cathodic mobility) core histones within 14 hr at the doses that we chose for response studies (figure 5a). As late as 34 hr of treatment, concentrations of 5 mM butyrate and 200 μM SBHA had maintained similar levels of hyperacetylation. A dose response of butyrate performed in JCA1 cells in the additional presence or absence of MCPA, an inhibitor of short chain acylCoA dehydrogenase [29], indicated that only at the lowest bioactive butyrate concentrations (100-300 μM butyrate) was metabolic consumption significantly limiting to the cellular response (figure 5b). Another experiment to address the issue of consumption of butyrate is shown in figure 6 in which it is shown that refeeding butyrate-treated JCA1 cultures with fresh butyrate led paradoxically to an increased yield of viable cells at later times, despite persistence of growth inhibition and an ability of cells to resume growth upon refeeding to remove butyrate. Therefore, these results suggested that the continued replication of JCA1 was likely not due to a deficiency in hyperacetylation response, but rather a tolerance thereof.

Response to butyrate of JCA1 and TSUPr1 sublines of T24 bladder cancer cell line

JCA1 cells were apparently more tolerant of HDI treatment, in that they failed to growth arrest until after 48 hr of treatment (figure 1). In addition, these cultures exhibited lower HDI-induced depression in CFE after 24-48 hr butyrate treatment than did the prostate cancer-derived cell lines. However, rates of DNA replication largely cease by 24 hr (figure 7). Most interestingly, JCA1 cultures appear to resume thymidine incorporation after 30 hr of butyrate treatment, and such cultures maintain modest rates of

Figure 5. Histone acetylation analysis on HDI-treated JCA1 and DU145 cell lines.

Cells were treated as growing cultures (typically 50% of maximum surface coverage), by adding 1/3 volume of 4X concentrated butyrate (final concentration 10 mM), suberoylbishydroxamate (SBHA; final concentration 200 μM) or control medium. After the indicated time of treatment, detached cells were collected from the culture medium by centrifugation, while adherent cells were collected by scraping into cold medium. Combined cells were rinsed, lysed with Triton X-114 in MOPS-buffered KCl solution (chromatin isolation buffer) with protease inhibitors, and separated into particulates (containing chromatin) and soluble protein. The later was used to gauge the relative number of cells (and therefore histones) per treatment group. Histones were eluted from chromatin using protamine and urea, electrophoresed on Triton/acid/urea polyacrylamide gels, fixed with trichloroacetic acid and stained with Coomassie blue. For figure 5b, butyrate treatments were 10 mM for 24 hr, while SBHA treatments were 200 μM for 24 hr. For figure 5c, a numerical index was derived to compare the butyrate dose response for acetylation in the presence and absence of MCPA (an inhibitor of butyrate metabolism). This index was derived by measuring the relative optical density for each of the bands by transmission densitometry and NIH Image analysis, and using these values to derive a weighted average of acetylation for histone H4 species according to the following formula (H4Ac1 + 2xH4Ac2 + 3xH4Ac3 + 4xH4Ac4)/(H4Ac0 + H4Ac1 + H4Ac2 + H4Ac3 + H4Ac4).

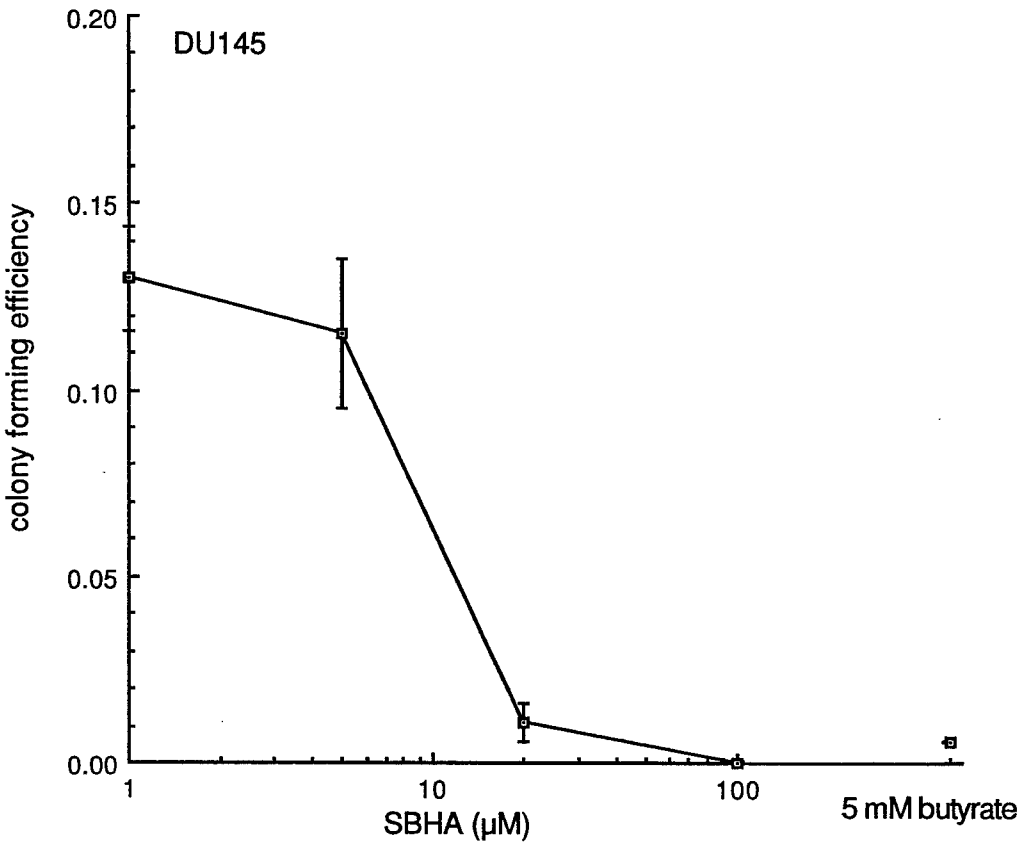


figure 5a. Comparison of the survival-inhibitory (CFE) effects of SBHA and butyrate on DU145

Cells were plated on plastic in D8F growth medium as in table 1 and treated with the indicated concentrations of SBHA or with 5 mM butyrate for 48 hr. After washing and replating non-adherent cells, dishes were reincubated for 17 days, fixed and stained with methylene blue. Data represents colony forming efficiencies (CFE), i.e., colonies per well divided by the input cell number as an average +/- standard deviation.

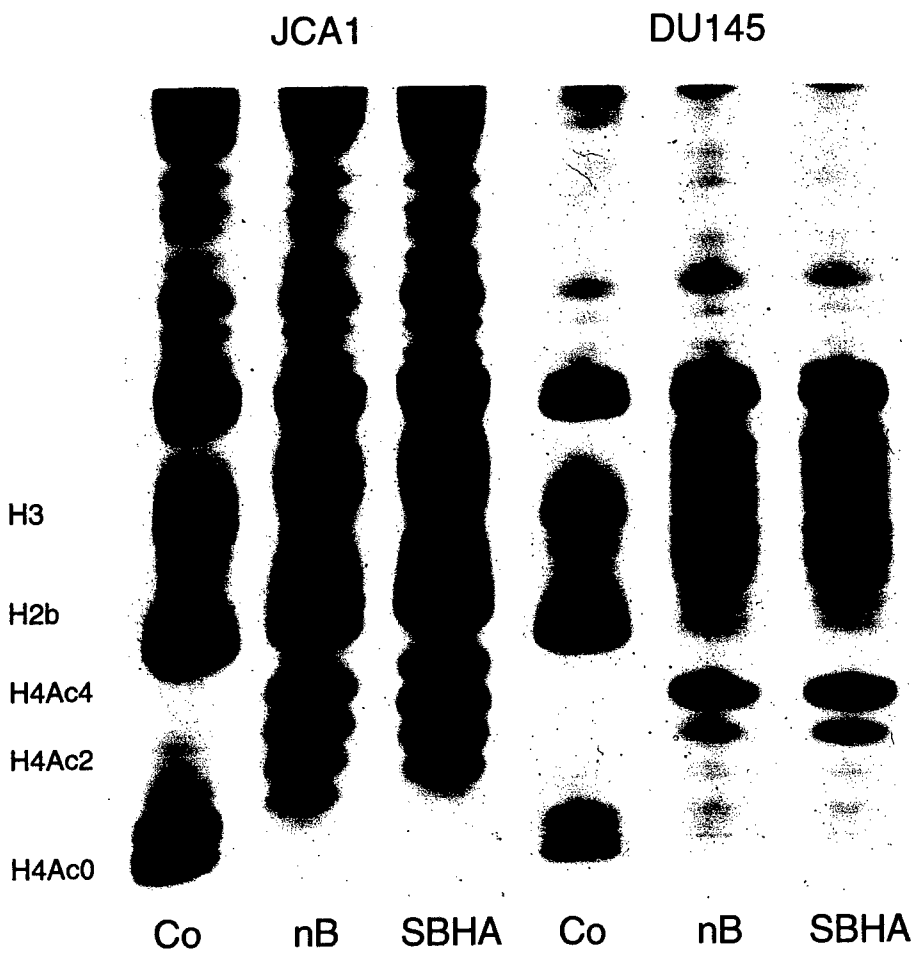


figure 5b. TAU-PAGE analysis of histone acetylation in JCA1 and DU145

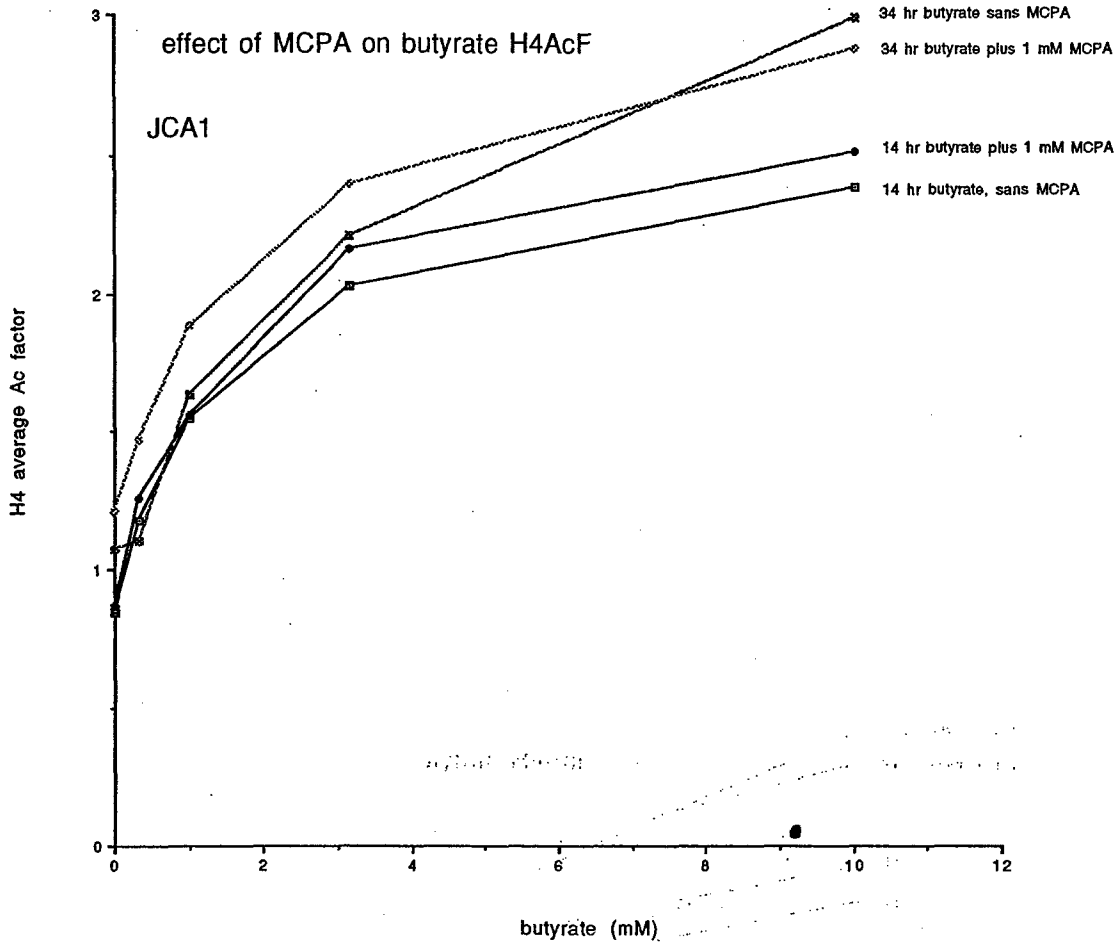


figure 5c. Effect of MCPA on butyrate-induced H4 acetylation by JCA1.

Methylenecyclopropanecarboxylic acid (MCPA) is a proximal inhibitor of butyrylCoA dehydrogenases. Replicate cultures were treated with a range of butyrate concentrations plus or minus MCPA at 1 mM. After 14 or 34 hr, cells were harvested for analysis of histones as above. Results demonstrate that metabolic consumption has a modest effect on long term effectiveness of low butyrate concentrations, but relatively little effect on actions of butyrate at concentrations over 3 mM in these bulk cultures.

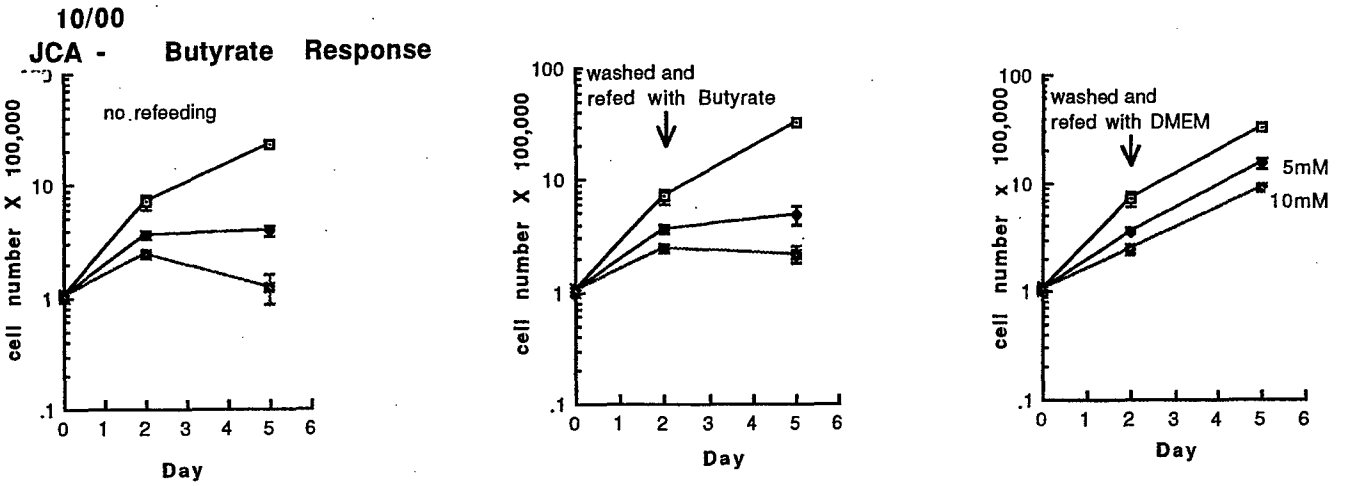


Figure 6. Effect of refeeding JCA1 cultures with fresh butyrate.

JCA1 cells were disaggregated and seeded into replicate dishes at 1 X 10^5 cells per 10 cm^2 dish and incubated for 2 days before beginning treatment. Butyrate was added as 1/3 volume of a 4X concentrate, and time zero counts were performed. Cultures were treated for 2 days with butyrate after which time they were either left unrefed (continuous treatment) or else were refed with fresh butyrate-containing or plain growth medium. After a further incubation, cells were collected by centrifugation of medium and trypsinization of adherent cells with trypsin/EGTA/dextran sulfate 8000. Viable cells were counted with a hemacytometer, using trypan blue to stain non-viable cells. Data are the average of three replicate determinations +/- standard deviation.

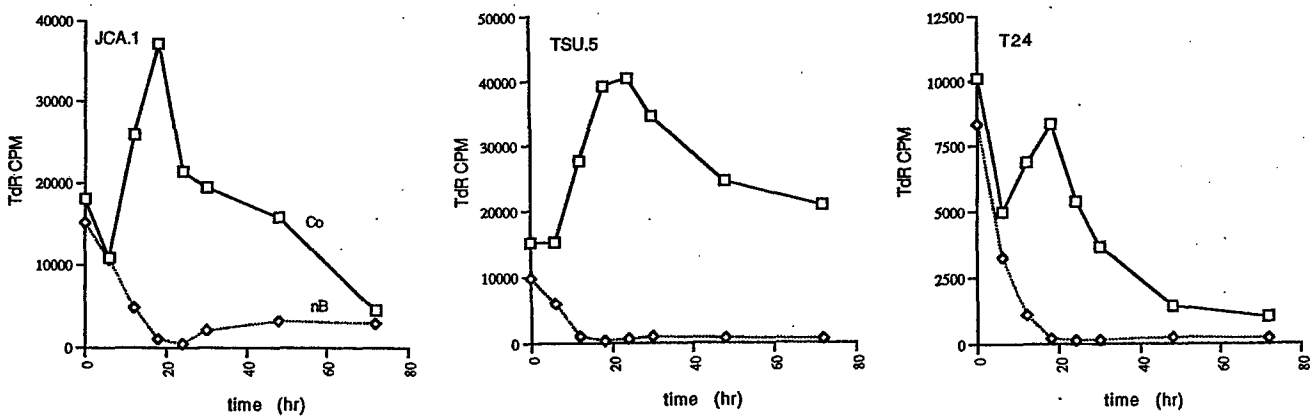


Figure 7. Effect of continuous butyrate treatment on DNA replication by T24 sublines.

Cells (cloned sublines of JCA1 or TSUPr1, and a low passage level culture of the T24 line) were treated as sparse growing cultures (typically 30-50% of maximum surface coverage), by adding 1/3 volume of 4X concentrated butyrate (final concentration 5 mM) or control medium. Labeling was performed on a set of three replicates at each successive 6 hr labeling/treatment period by adding [^{3}H]-thymidine (1 µCi/2 ml; 10 µM final total [thymidine]). Cells were harvested by centrifuging the spent medium and scraping adherent cells into PBS with 10 mg BSA/ml. The combined cells and BSA carrier were fixed in 5% trichloroacetic acid, collected on glass fiber depth filters (Whatman GF/C) and rinsed with 70% ethanol. Macromolecules were hydrolysed in formic acid and counted by liquid scintillation spectrometry. Data represent the average of three determinations. The JCA1 results are typical of data collected on two separate clones.

incorporation at least until 72 hr of treatment. We have not yet determined whether or not the cells that incorporate thymidine subsequently finish S phase or instead undergo apoptosis. Unlike other butyrate-treated cell lines, JCA1 neither arrests in G2 (figure 3) nor dies thereafter, two fates of HDI-treated cells correlated with the presence and absence, respectively, of HDI-induced p21cip1 expression [1, 2]. In contrast to JCA1, the TSUPr1cell population appears fully non-replicative over the course of a 2 day butyrate treatment period, exhibits a prominent G2 phase arrest (figure 3), and like T24 itself fails to exhibit the delayed, spontaneous resumption of DNA replication that characterizes JCA1. Both JCA1 and TSUPr1 cultures thereafter exhibit progressively greater proportions of apoptotic cells (subG1) unless HDI treatment is terminated by refeeding.

We have been unable to reconcile the 4-5 fold increase in cell number evident from hemacytometer counts (figure 1b-d) with the profound, rapid inhibition of tritiated thymidine incorporation. It remains possible that the thymidine incorporation data, obtained thus far on only 2 of 8 available clones, was not representative of the non-cloned population used for the growth curves and DNA-PI analysis. It is also possible that some apoptotic cell fragments were counted as cells, although this was said to be unlikely, and my technician was extraordinarily careful.

The nature of butyrate-induced cell death in DU145, JCA1 and PC3.

The DU145 and PC3 prostate cancer cell lines, both poorly differentiated but distinctly different in apparent growth requirements, originated as brain metastasis and bone metastasis, respectively. PC3 obviously requires accumulation of some autocrine factor, or juxtaposition of stromal cells or their products. DU145, on the other hand, is genuinely autonomous, and appears to be growth inhibited by some product of stromal cells. The responses of these two cell lines to butyrate are also quite distinguishable. The loss of DU145 cell numbers that occurs between 24 and 48 hr of butyrate or SBHA treatment largely has little resemblance to apoptosis – little cleavage of PARP or PKCepsilon was evident considering the magnitude of loss of CFE. In fact, the JCA1 cell line exhibits more apoptotic protein cleavage and subG1 cell accumulation than DU145, yet suffers losses of approximately 50% after 48 hr treatment, compared with 80% for DU145.

As to the nature of the growth arrest state in PC3, subG1 cells (PI stain) and apoptotic cleavage fragments (24 or 48 hr) of either PARP or PKCepsilon are less prominent than in each of the other cell lines. Total inhibition of PC3 growth can be obtained by seeding them in conventional medium at a low density and treating them in such a 'colonial' state. We found that both basal CFE and butyrate refractoriness improve in cultures of increasing seeding density or coculture with bone-derived stromal cells. It is possible that for cells such as PC3, the growth limitation is magnified during an otherwise tolerable HDI-induced growth arrest. Interestingly, with extended passage a variant cell line was obtained that had a more mesenchymal appearance, better growth at low densities and a basal CFE of nearly 25% (table 2). This cell line remained fully susceptible to HDI-induced growth arrest and CFE reduction. Although we haven't compared the recoverability or apoptosis susceptibility of this variant, we are intrigued by the possibility that these cells may allow us to dissociate certain CFE-enhancing effects of stromal cell coculture from effects on recovery from HDI-induced cytostasis.

The nature of mezerein-induced and butyrate-induced LNCaP cell death

The LNCaP cell line suffers major losses of CFE when treated for 48 hr with butyrate and assayed for CFE. Similarly, this cell line is highly susceptible to CFE loss when treated for 48 hr with protein kinase C activators such as mezerein or TPA. Although several reports have ascribed such persistent lack of proliferation to apoptosis, our examination of 24 - 48 hr mezerein-treated cultures for morphological evidence of apoptosis, sub-G1 DNA-containing cells on cytofluorograms, or apoptotic cleavage of cellular proteins revealed relatively little evidence for early apoptosis. For example, apoptotic fragmentation of either PARP or PKC epsilon, two proteins useful for this purpose due to retention of fragments in particulate fractions of dead cells, was minimal within 48 hr for LNCaP cells treated with

either butyrate, TPA or mezerein (figure 2). While we recovered all cells, both non-adherent and attached, by direct lysis into SDS with alkylators and other protease inhibitors for these experiments, the growth curves clearly show that viable cell loss was occurring prior to 48 hr of butyrate treatment. While a much higher level of apoptotic proteolysis was seen after butyrate treatment than after mezerein treatment, in neither case did such activity reach a level that could account for the reduction in CFE that was effected by either of these transitory treatments. The combination of the two agents led to a supraadditive increase in apoptotic proteolysis, but because the mechanism of committment to non-replication effected by each agent remains unclear, statistical analysis of this interaction is premature. We believe that additional investigation of TPA-treated LNCaP cultures for evidence of terminal differentiation may reveal that apoptotic cell death occurs well after the cells have committed to non-replication, as we have previously noted for VACO 10MS colon cancer cells terminally differentiating under the influence of TPA [30].

d. Potential role of cyclin and CKIkip proteins in HDI tolerance.

Cyclin/CKIkip in butyrate tolerance and refractoriness to HDI-induced G2 arrest of JCA1

Much of the growth inhibitory action of butyrate and other HDI's has been ascribed to p21cip1 [10, 12]. However, these studies have also suggested that p21-deficient cells are at increased risk of HDI-induced death, and are unstable in HDI-induced G2 arrest [1, 2]. Several investigators have pointed to a role of p21cip1 in G2 arrest itself [17, 18]. We found, however, that JCA1 was refractory to the G2 arresting effects of butyrate in the face of p21cip1 expression considerably higher than that in cells durably arrested in G1 or G2. The finding that JCA1 cells appeared to subsequently arrest with a G1 complement of DNA led us to suppose that G2 arrest was neither necessary nor sufficient for tolerance of HDI treatment. Current thinking holds that cells with hyperacetylated chromatin which fail to arrest prior to the G2/M transition are at a high risk of apoptosis [1, 2].

In an effort to determine whether the unusual HDI resistance of JCA1 could be correlated with major deficiencies in expression of cyclin-related growth regulatory proteins, we examined the composite of cyclin and CKIkip proteins in butyrate-treated and control cell cultures of JCA1 and TSUPr1 cell lines. As shown in figure 8, butyrate treatment led within 24 hr to an increase in cyclins E (and D3, not shown), and reductions in cyclins A, D1 and B for both TSUPr1 and JCA1. Among the CKIkip proteins, p21cip1 and p57kip2 were each expressed at a higher level in these cell lines after butyrate treatment.

The period of time when evidence of apoptotic death is increasing, between 24 and 72 hr of butyrate treatment, also corresponds to the time when JCA1 cultures are exhibiting a modest increase in thymidine incorporation (see figures 3 and 7). Because histone acetylation remains high at least until 34 hr, we assume that the cells are remaining responsive to HDI effects. The spontaneous rise in thymidine incorporation after a near cessation suggested that mediators of HDI-induced G1 arrest were unexpectedly decaying or becoming less active, or were failing to be expressed at later times. An examination of JCA1 cells for changes in expression of the cyclin and CKIkip proteins over time revealed a progressive decline in abundance of p21cip1 and p57kip2 after a maximum at 24 hr (figure 9a and e). At the same time we noted a progressive rise in levels of CKIkip-binding proteins, cyclins E (figure 9a) and D3. Thus, not only were butyrate-treated JCA1 cells exhibiting a decline in p21cip1 protein *per se*, but also a decline in the ratio of p21cip1 to cyclin E. This was occurring in JCA1 and TSUPr1(figure 9b) at the time that apoptosis in both TSUPr1 and JCA1 was rising (24-72 hr). The ratio of p21cip1 to cyclin E, which is likely to be related to the abundance of cyclin-free p21cip1, could be near that of untreated cells by 72 hr of butyrate treatment. An examination of DU145 in a similar manner

Figure 8. Effect of 24 hr butyrate treatment on expression of cyclins and CKIkips by T24 derivatives, and by DU145 and its ND1 subline.

Cells were treated as growing cultures (typically 50% of maximum surface coverage), by adding 1/3 volume of 4X concentrated mezerein (final concentration 100 nM), butyrate (final concentration 5 mM) or control medium (0.025% final [ethanol]). After the indicated time of treatment, detached cells were collected from the culture medium by centrifugation, while adherent cells were lysed in place with SDS, N-ethylmaleimide, EDTA and other protease inhibitors in a tris-buffered saline. The combined lysates were stored at -20°C and assayed for total protein (BCA). Aliquots corresponding to equal protein (50-100 µg/ml), reduced with mercaptoethanol and boiled, were electrophoretically separated using a Laemmli discontinuous buffer in an SDS polyacrylamide gel. Separated proteins were electrophoretically transfered from the gel to PVDF membranes. After blocking non-specific surfaces of the membranes using skim milk and BSA, specific proteins and their cleavage products were localized using antibodies to a) p21cip1 (PMNG 556431, MC), p27 (SC1641, MC) or p57kip2 (SC1040, PC), or b) cyclin D1/2 (SC718, PC), cyclin E (SC247, MC), cyclin A1/A{2} (SC751, PC), or cyclin B (SC245, MC) and peroxidase-conjugated antibodies to mouse (MC) or rabbit (PC) immunoglobulin. Immunoperoxidase was detected and recorded on Xray film using ECL luminol reagent (Amersham). The result for p21cip1 Mr appears to include an artifact corresponding to the difference between the SDS front (near 18 kDa) and the salt front. Each set of determinations were a single exposure of a blot/gel analysis of lysates from each of the cell lines treated for 24 hr with butyrate (5 mM) or control medium.

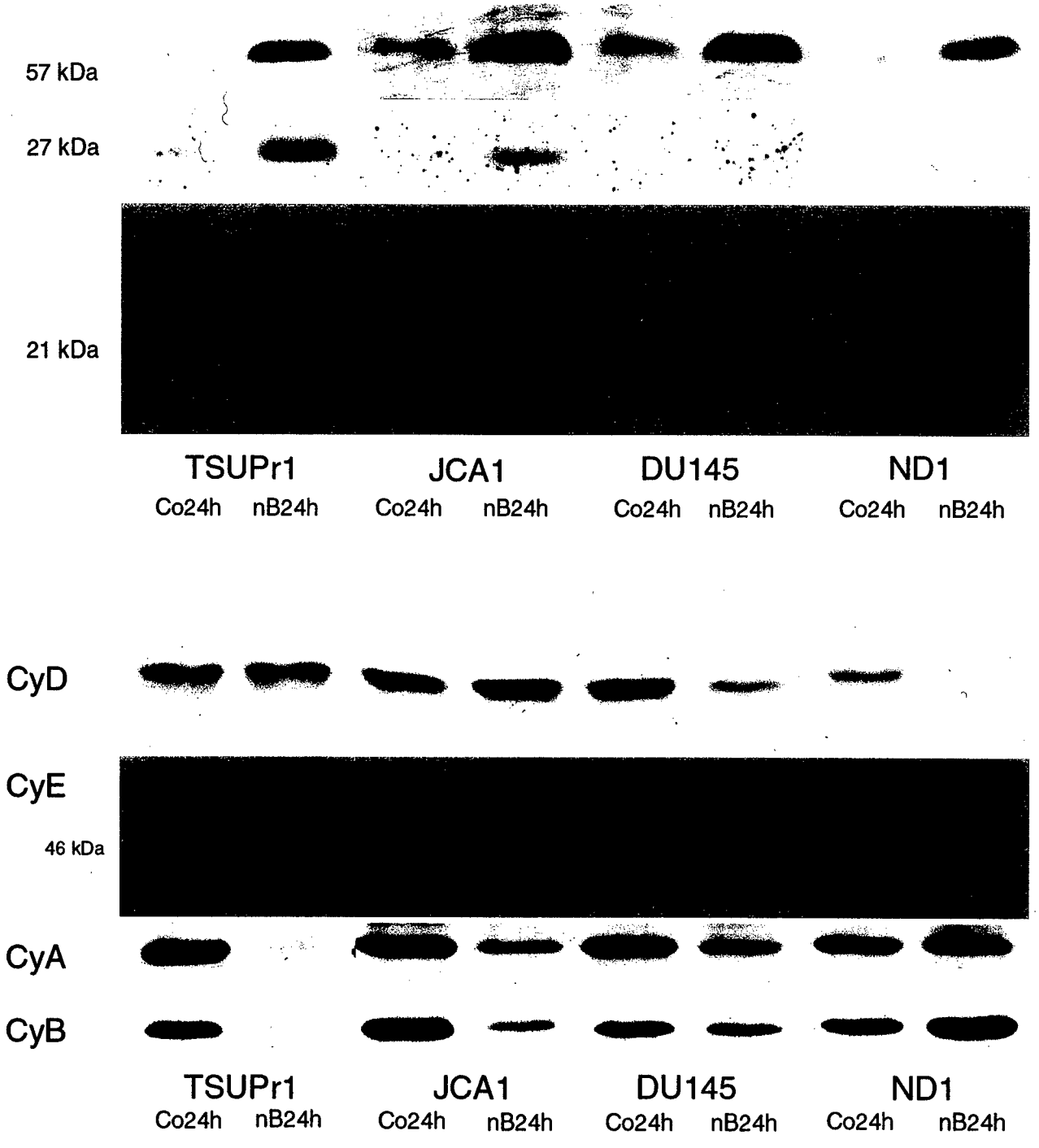


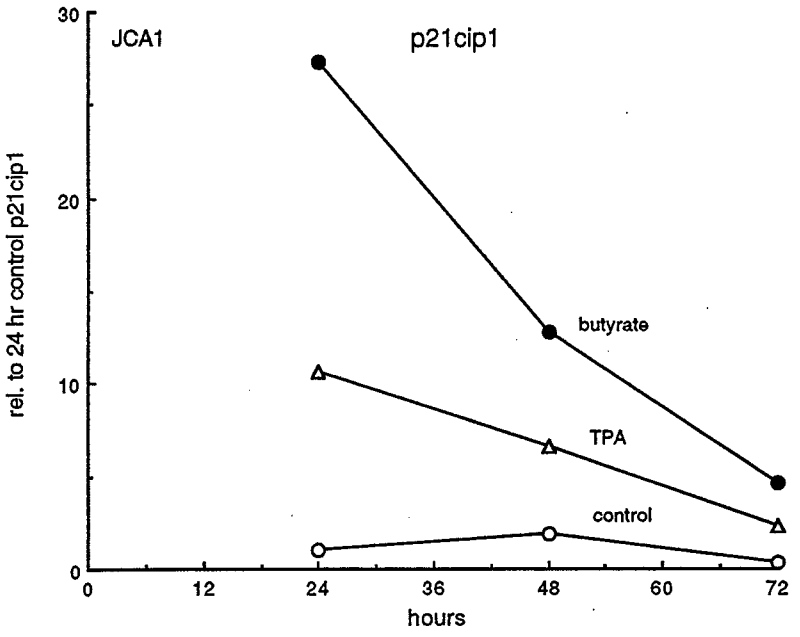
Figure 8. Effect of 24 hr butyrate treatment on expression of cyclins and CKIkips by DU145 and T24 derivatives.

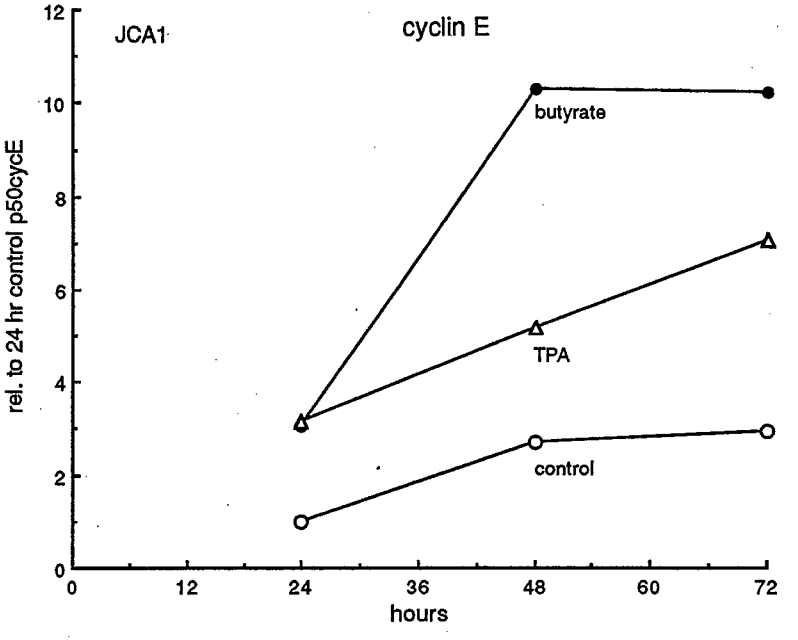
Figure 9. Changes in abundance of cyclin and CKIkip proteins during treatment.

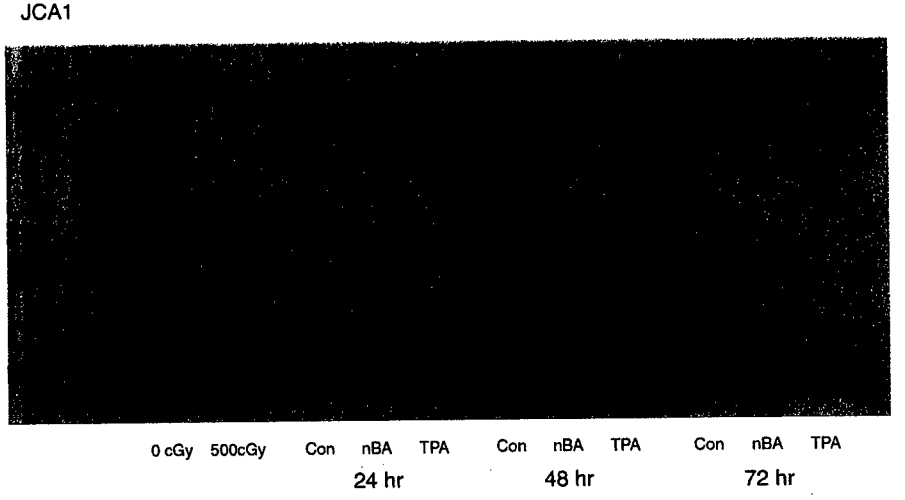
Cells were treated as growing cultures (typically 50% of maximum surface coverage), by adding 1/3 volume of 4X concentrated mezerein (final concentration 100 nM), butyrate (final concentration 5 mM) or control medium (0.025% final [ethanol]). After the indicated time of treatment, detached cells were collected from the culture medium by centrifugation, while adherent cells were lysed in place with SDS, N-ethylmaleimide, EDTA and other protease inhibitors in a tris-buffered saline. The combined lysates were stored at -20°C and assayed for total protein (BCA). Aliquots corresponding to equal protein (50-100 µg/ml), reduced with mercaptoethanol and boiled, were electrophoretically separated using a Laemmli discontinuous buffer in an SDS polyacrylamide gel. Separated proteins were electrophoretically transfered from the gel to PVDF membranes. After blocking non-specific surfaces of the membranes using skim milk and BSA, specific proteins and their cleavage products were localized using antibodies to p21cip1 (PMNG 556431, MC), p27 (SC1641, MC) or p57kip2 (SC1040, PC), or cyclin E (SC247, MC), followed by peroxidase-conjugated antibodies to mouse (MC) or rabbit (PC) immunoglobulin. Immunoperoxidase was detected and recorded on Xray film using ECL™ luminol reagent (Amersham). Darkened areas on these films were semiquantitated using a transmission scanner and computer to collect digital tiff files for analysis by the NIH Image 1.61 program. None of the images were saturated for more than 5% of the analyzed bands. Backgrounds were subtracted as manual straightline estimates corresponding to local averages. Values plotted in these graphs correspond to the relative optical density of peaks corresponding to the major band near the consensus Mr reported in the literature and by the antibody supplier.

fig. 9a. Abundance of p21cip1 and cyclin E in JCA1 treated 24-72 hr with butyrate or TPA.. Cell lysates (control for irradiation, 500 cGy irradiated, daily controls for TPA/butyrate, 5 mM butyrate or 100 nM TPA) were analysed sequentially for both proteins on the same blot and Xray film. Densitometry data are normalized to the value of the specific protein band from the 24 hr control sample. Raw digital photograph of moderately long exposure and densitometry results obtained from the combination of long and short exposures, with densitometry values for saturated signals multiplied by a factor that equalized the p21cip1 and cyclin E values for the (nonsaturated) 24 hr TPA-treatment samples.

fig. 9a. Abundance of p21cip1 and cyclin E in JCA1 treated 24-72 hr with butyrate or TPA







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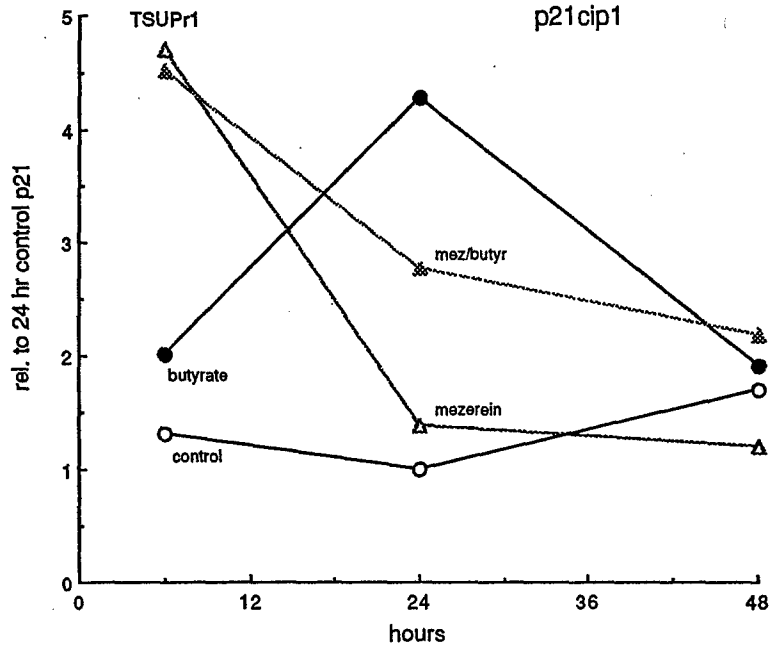


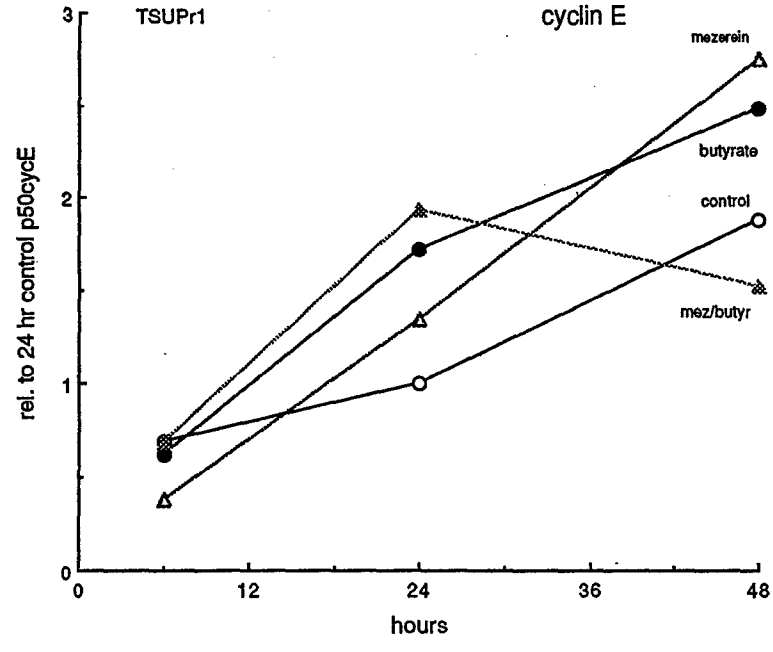


fig. 9b. Abundance of p21cip1 and cyclin E in TSUPr1 cells treated for up to 48 hr with butyrate or 100 nM mezerein or the combination of the two agents. Densitometry data are normalized to the value of the specific protein band from the 24 hr control sample.

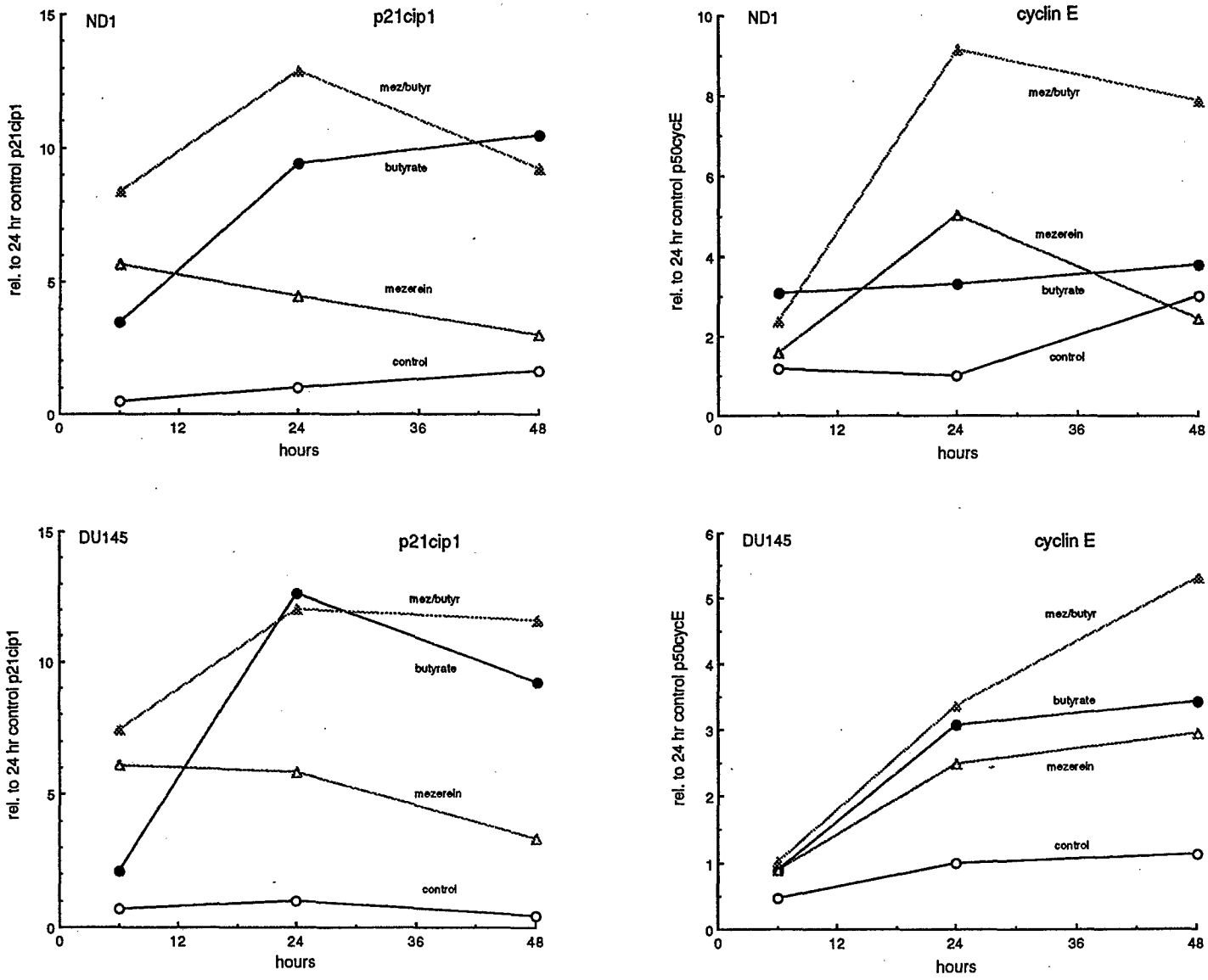
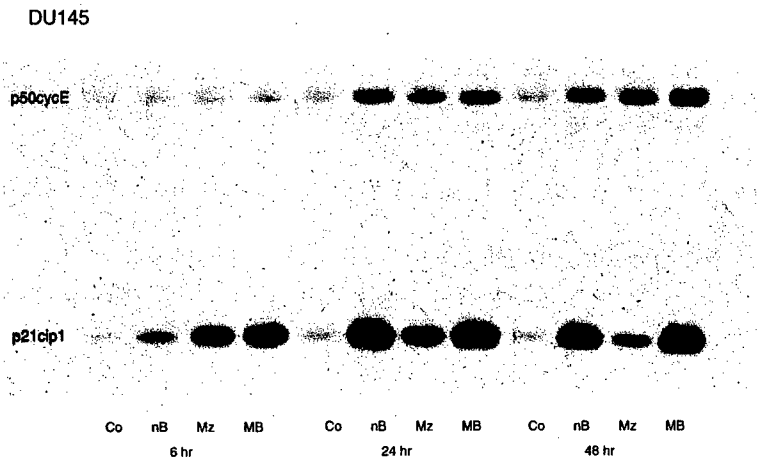


fig. 9c. Abundance of p21cip1 and cyclin E in ND1 and DU145 cells treated for up to 48 hr with butyrate or 100 nM mezerein or the combination of the two agents.
Densitometry data are normalized to the value of the specific protein band from the 24 hr control sample.



blot used for figure 9c DU145 densitometry. Co, control; nB, 5 mM butyrate, Mz, 100 nM mezerein, MB, 5 mM butyrate and 100 nM mezerein. Samples were analysed concurrently with antibodies to both p21cip1 and cyclin E. The results illustrate that, unlike the results for JCA, p21cip1 levels remain elevated at 48 hr. To the extent that blotting efficiencies and antibody saturations were similar for both proteins in the two assays, these results indicate that DU145 exhibits a less exuberrant induction of both p21cip1 and cyclin E than does JCA1. Relative to the signal associated with cyclin E at 48 hr, p21cip1 levels in DU145 cells appear quite strong. Of course these inferences would never stand up to review, assuming that one p21cip1 protein / monoclonal antibody complex relays the same signal as one cyclin E protein / monoclonal antibody complex. Nonetheless, the hypothesis is supported by several other pieces of evidence.

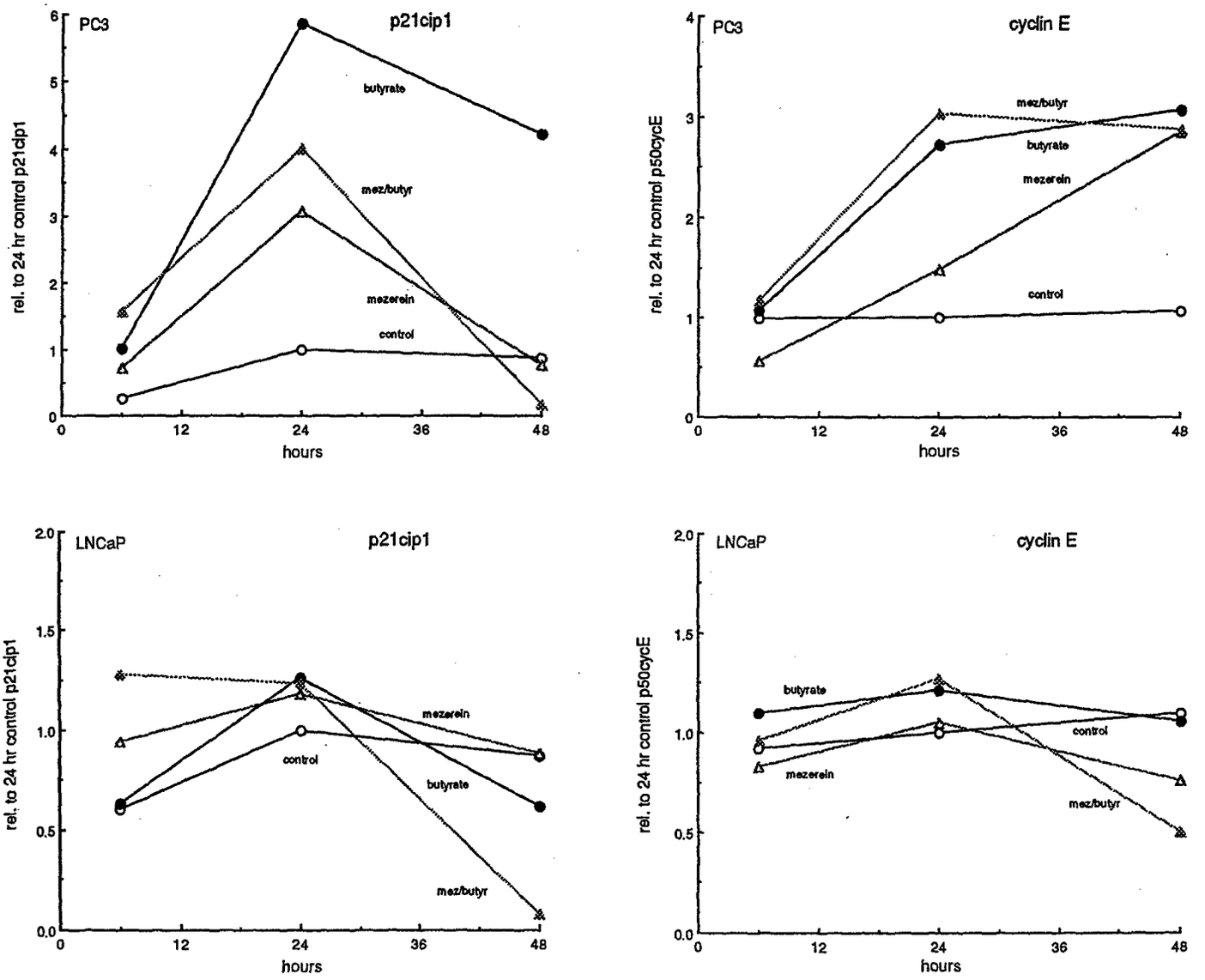


fig. 9d. Abundance of p21cip1 and cyclin E in PC3 and LNCaP cells treated for up to 48 hr with butyrate or 100 nM mezerein or the combination of the two agents.
Densitometry data are normalized to the value of the specific protein band from the 24 hr control sample.

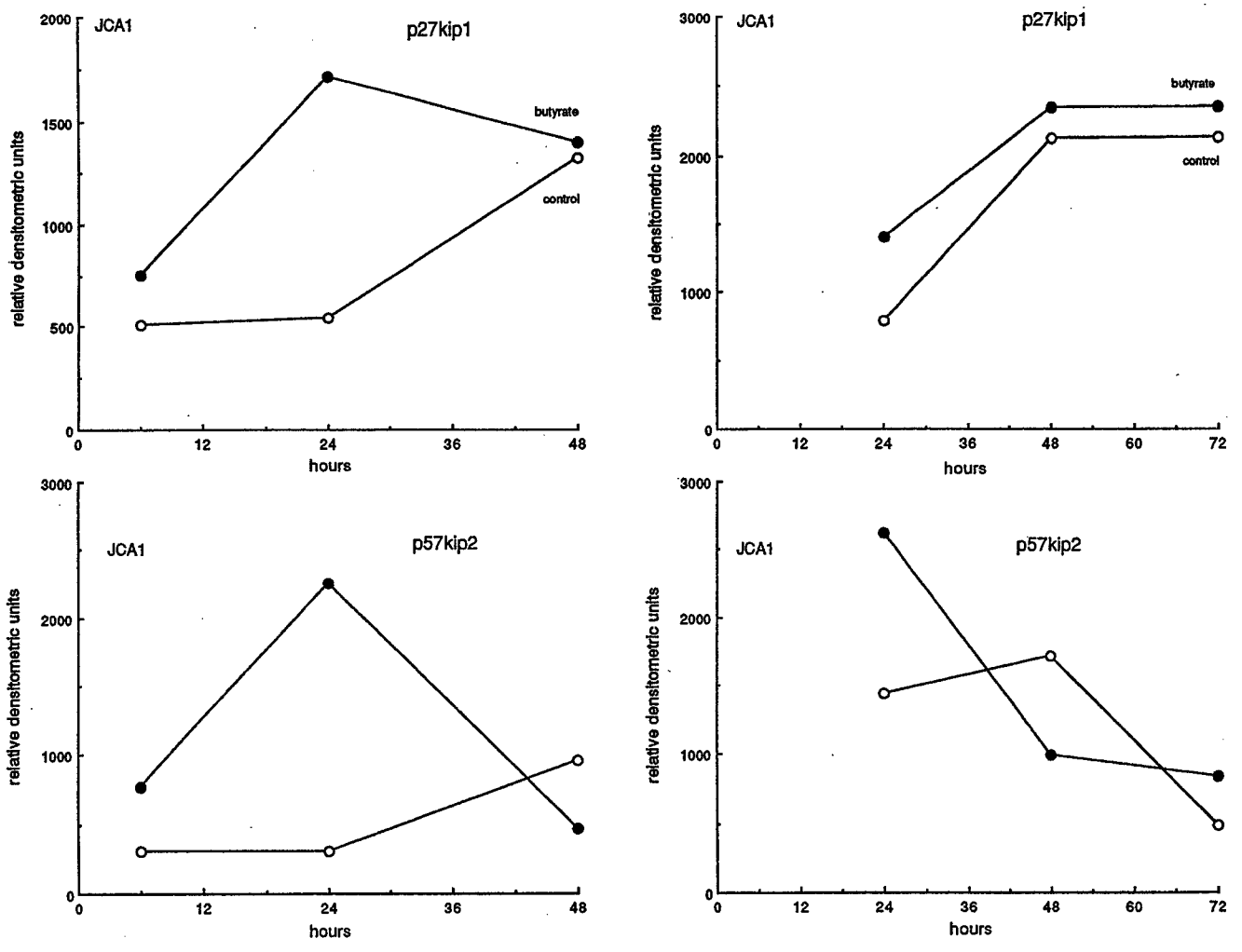


fig. 9e. Abundance of p27kip1 and p57kip2 in JCA1 cells treated for up to 72 hr with butyrate. The two profiles on the left and the two on the right were from separate experiments.

indicated that these cells not only expressed a lower level of cyclin E relative to p21cip1, but maintained a similar ratio of p21cip1 to cyclin E proteins at 24 hr and 48 hr (figure 9c).

These results led us to hypothesize that uncomplexed CKIkip proteins p21cip1 and p57kip2 (figure 9e) might be playing a positive role in HDI tolerance irrespective of cell cycle phase (the hypothesis behind our failed VA Merit Review application). Interestingly, such a mechanism obviates the alternative hypothesis, that the JCA1 cell line is uniquely tolerant of cell division involving hyperacetylated chromatin, although such unusual behavior could normally require an apoptosis suppressor, an activity such as we are postulating for p21cip1 and p57kip2. Because apoptosis was also associated with a rise in rates of thymidine incorporation, it is tempting to hypothesize that survival-promoting actions of CKIkip proteins were primarily typical CDK inhibitory actions, preventing rereplication of chromatin that has been segregated while hyperacetylated. In order to link such relaxation of G1 arrest with apoptosis, we would need to be able to demonstrate that apoptotic JCA1 cells had on average incorporated an intermediate amount of tritiated thymidine. Such DNA replicative activity could be ascribed to cyclin E/CDK2 levels having risen above the threshold needed for initiation of DNA replication. Presumably, active cyclin E/CDK2 was in addition to cyclin/CDK complexes bearing CKIkip proteins, signifying the absence of free p21cip1. The finding of similar frequencies of apoptosis in TSUPr1, which does not exhibit increases in thymidine incorporation yet undergoes apoptosis and reciprocal changes in p21cip1 and cyclin E argues against cell cycle phase-specific mechanisms of apoptosis triggering and p21cip1 protective action.

Such results may be complementary to the findings of Burgess et. al. [2], who modified an earlier hypothesis of G2 phase bypass as a trigger for HDI-induced apoptosis [1]. These investigators found that p21cip1-deficient cells that are blocked from exiting G0/G1 by overexpression of the p16 CDK inhibitor, or stalled at the G1/S boundary by hydroxyurea inhibition of DNA replication, die within 48 hr of HDI treatment even though they do not pass G2 [2]. These studies and our own observations on TSUPr1 and JCA1 cultures suggest that HDI-induced cell death is related to some universal apoptosis-susceptibility of histone deacetylase inhibited cells that is forestalled by p21cip1 and/or p57kip2 accumulation.

The realization that levels of uncomplexed p21cip1 might correlate with both HDI-tolerance and inhibition of thymidine incorporation by JCA1 and TSUPr1 leaves open the question of how TSUPr1 differs from JCA1 in competence for G2 arrest. None of the differences in cyclin or CKIkip protein abundance changes would alone suggest a reason for the two cell lines to differ in G2/M transit in such a marked fashion. An immunohistochemical study of p21cip1 expression by control and HDI-treated cells in cultures of JCA1 and TSUPr1 revealed an additional difference relating to subcellular localization of this protein. In logarithmically growing cultures of each cell line, presumptive basal expression leads to decoration of nuclear structures in 4/5 of the cells but cytoplasmic structures in the remainder (figure 10b&c). Cells with this later expression have characteristic round morphologies, little spreading on the coverslip, and stain-exclusion of structures that may correspond to segregated chromosomes or nascent nuclei. The finding that such cells are prominent in 24 hr HDI-treated JCA1 and control TSUPr1 (figure 10e) but not 24 hr HDI-treated TSUPr1 (figure 10g) suggests that such cells may be G1 phase cells. Their rarity in 48 hr butyrate-treated cultures suggests that that such cells may have succumbed to apoptosis as a first wave of such death. The presence of intense p21cip1 immunostaining in a cytoplasmic location after HDI treatment is provocative given the association of such localization in death-protected differentiating cells [16]. However, the consequences may be trivial for JCA1, in which such protein presumably meets the same fate as bulk p21cip1 given the low abundance of such cells from 48 hr HDI-treated cultures (figure 10d). When we examined 48 hr HDI-treated TSUPr1 cells for those with extremely low p21cip1 expression, we found that such cells also exhibited degradative changes (shrinkage, fragmentation and blebbing) (figure 10h).

Figure 10. Immunocytochemical localization of p21cip1 in butyrate treated JCA1 and TSUPr1.

Cells were plated on polyethyleneterephthalate (Thermanox™) coverslips, grown for 2 days and then treated as growing cultures with 5 mM butyrate or control growth medium. At the indicated times, coverslips were dried and fixed in methanol. Cells were stained with a monoclonal antibody to p21cip1 (Pharmagen 556431, clone SXM30) and visualized using an immunoperoxidase secondary antibody ABS method (Vectastain™) with benzidine chromogen. Images were collected with a digital camera.

figure 10a-d. Immunocytochemical localization of p21cip - JCA1.
a. 24 hr control; b. 6 hr butyrate; c. 24 hr butyrate; d. 48 hr butyrate

figure 10e-h. Immunocytochemical localization of p21cip - TSUPr1.
e. 24 hr control; f. 6 hr butyrate; g. 24 hr butyrate; h. 48 hr butyrate

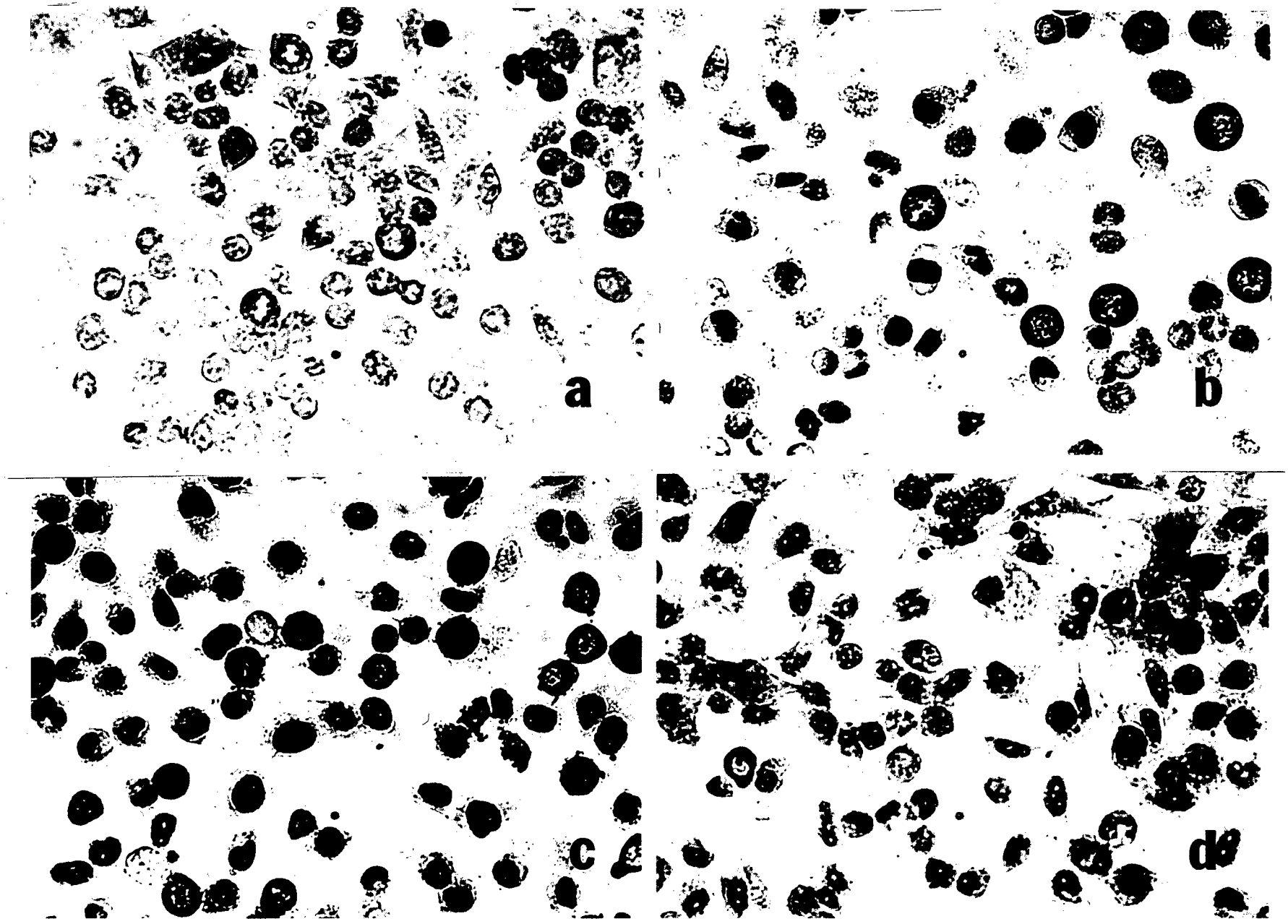


figure 10. Immunocytochemical localization of p21cip - JCA1.
a. 24 hr control; b. 6 hr butyrate; c. 24 hr butyrate; d. 48 hr butyrate

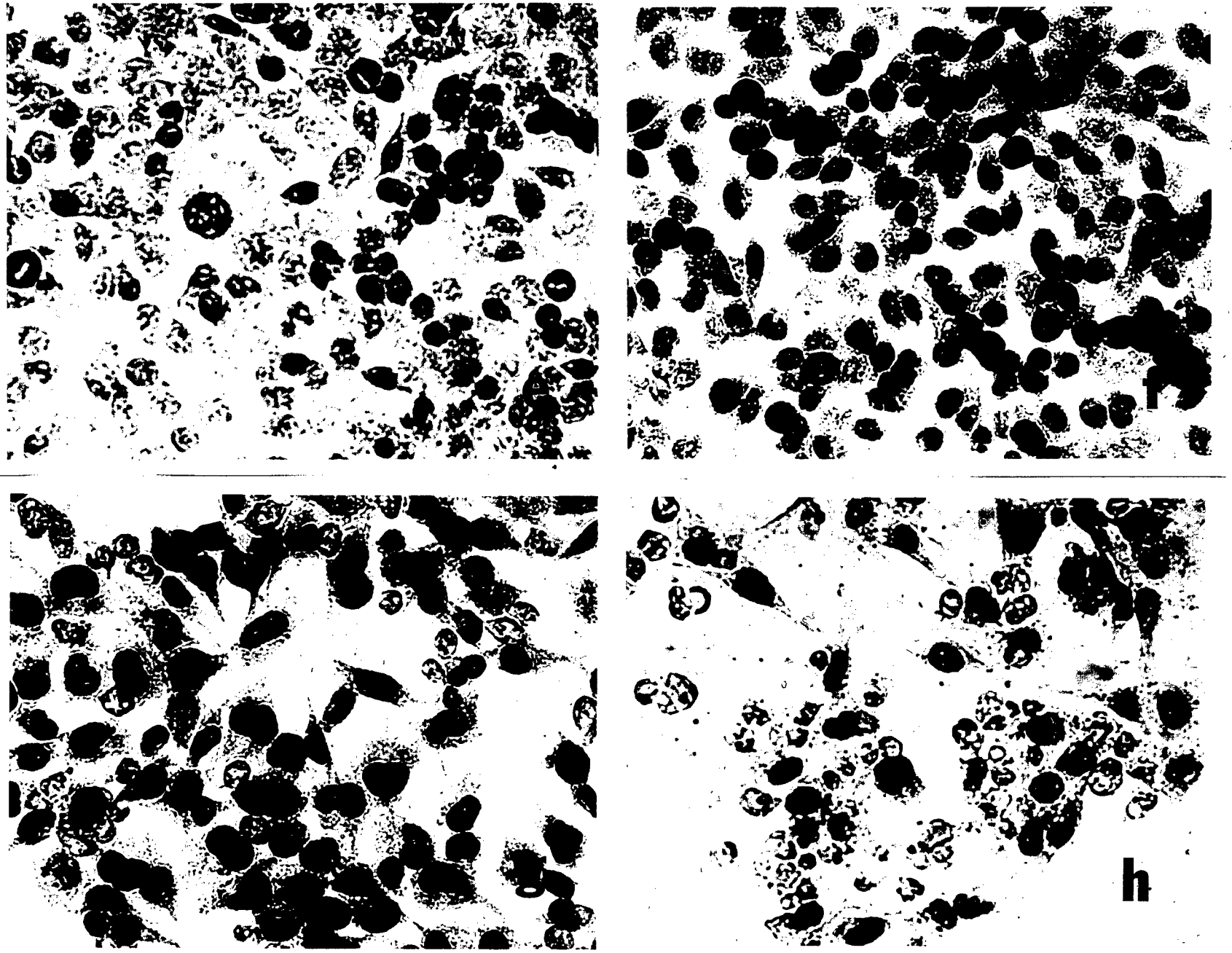


figure 10. Immunocytochemical localization of p21cip - TSUPr1.
e. 24 hr control; f. 6 hr butyrate; g. 24 hr butyrate; h. 48 hr butyrate

Distinctions between DU145 and ND1 responses to butyrate.
This pair of phenotypically distinguishable cell lines, in which the ND1 subline arose by inadvertent cloning of DU145 into a primary culture of prostate tumor, has been shown repeatedly to resemble one another. Specifically, both lines are known to express high levels of a mutant p53 protein and negligible bax protein [27]. Also not surprizingly the cell lines differ relatively little in proportionate survival after HDI treatment, and respond to HDI with changes in CKIkip and cyclin protein abundance. However, the ND1 line does not suffer such an immediate reduction in viable cell numbers during HDI treatment and has a somewhat enhanced survival (greater CFE after treatment). Furthermore, ND1 suffers much less from the CFE-inhibitory effects of normal bone stroma coculture that marks DU145 as different from each other cell line (table 1). The ND1 cell differs from DU145 by a few molecular features of butyrate responsiveness: 1) less prominent loss of cyclin A species. 2) unusual and substantial HDI-induced increase in cyclin B species. 3) reduced levels of cyclin D1 and D2, with additional prominent loss during butyrate treatment. 4) greater accumulation of cyclin E and more prominent cleavage fragments and high molecular weight immunoreactive species. Overall, however, differences of HDI responsiveness between ND1 and DU145 were relatively minimal, so this pair of cell lines has received relatively little additional study relative to HDI action. However, the accumulation of cyclin B by HDI-treated ND1 cells was of a sufficiently peculiar nature that this finding may justify a cytometric analysis of PI-DNA and then possibly publication. The finding that ND1 cells express little sensitivity to bone stroma-suppression of growth may provide additional rationale to continue study of this adventitious contaminant..

Distinctions among unique prostate cancer cell lines (PC3, LNCaP and DU145) in HDI responsiveness
One of the first distinctions that was noted among CKIkip protein expression results (figure 11a) was the high basal expression of p21cip1 and p27kip1 by LNCaP, and high basal expression of p57kip2 by PC3 cells. In the case of p21cip1 and p57kip2, such basal expression by LNCaP and PC3, respectively, was associated with a lack of further induction during butyrate treatment. Because cyclins E and D3 were accumulating under the influence of the HDI (figures 9d and 11), this limited induction may have corresponded to a net decrease in cyclin-free p21cip1 in LNCaP and free p57kip2 protein in PC3. We are planning to test for normalization of both basal CKIkip protein expression (e.g., lower basal p21cip1 abundance in LNCaP and p57kip2 in PC3) and for a greater HDI-inducability of these proteins in an environment that better supports survival, such as coculture with bone stroma.

c. Bone stroma / prostate cancer coculture - growth support and HDI-tolerance.

Cell line establishment.
Our anticipation of success in deriving new prostate cancer cell lines was based on our work with colorectal cancer cell lines, as well as published reports. We would at the outset predict that culture competent cells would be limited to those individuals who will eventually recur with metastatic disease. The 45% success rate we reported for cell line establishment from colorectal cancers [31] included 70% from primary cancers of persons with synchronous metastatic disease (Dukes' stage D), 40% from primary cancers of lymph node-involved patients (Dukes' stage C) and 0% success for colonic wall-limited cancers (Dukes' stage A or B). Although prostatectomy is supposedly limited to persons with no evidence of extraprostatic cancer, a significant proportion of men who have this surgery recur with distant disease. Furthermore, 6 individual prostate cancer cell lines were said to be derived from primary prostate cancers. However, during 2001, each of these cell lines has been found to be a product of contamination by other prostate and bladder cancer cell lines. The findings that the 'primary prostate cancer'-derived cell lines ALVA 31 & 41 and PPC1 are PC3, that ND1 is DU145, and that TSUPr1 and JCA1 are T24 bladder cancer indicates that prostate cell line establishment success to date has been limited to extraprostatic disease. Our lack of success after 43 attempts at culture of primary prostate cancers was thus consistent with biological reality and good technique, and with the clinical practice of

Figure 11. Cyclin and CKIkip protein expression by prostate cancer cell lines.

Cells were treated as growing cultures (typically 50% of maximum surface coverage), by adding 1/3 volume of 4X concentrated mezerein (final concentration 100 nM), butyrate (final concentration 5 mM) or control medium (0.025% final [ethanol]). After the indicated time of treatment, detached cells were collected from the culture medium by centrifugation, while adherent cells were lysed in place with SDS, N-ethylmaleimide, EDTA and other protease inhibitors in a tris-buffered saline. The combined lysates were stored at -20°C and assayed for total protein (BCA). Aliquots corresponding to equal protein (50-100 µg/ml), reduced with mercaptoethanol and boiled, were electrophoretically separated using a Laemmli discontinuous buffer in an SDS polyacrylamide gel. Separated proteins were electrophoretically transfered from the gel to PVDF membranes. After blocking non-specific surfaces of the membranes using skim milk and BSA, specific proteins and their cleavage products were localized using antibodies to a) p21cip1 (PMNG 556431, MC), p27 (SC1641, MC) or p57kip2 (SC1040, PC), or b) cyclin D1/2 (SC718, PC), cyclin E (SC247, MC), cyclin A1/A{2} (SC751, PC), or cyclin B (SC245, MC) and peroxidase-conjugated antibodies to mouse (MC) or rabbit (PC) immunoglobulin. Immunoperoxidase was detected and recorded on Xray film using ECL luminol reagent (Amersham). The result for p21cip1 Mr appears to include an artifact corresponding to the difference between the SDS front (near 18 kDa) and the salt front. Each set of determinations were a single exposure of a blot/gel analysis of lysates from each of the cell lines treated for 24 hr with butyrate (5 mM) or control medium.

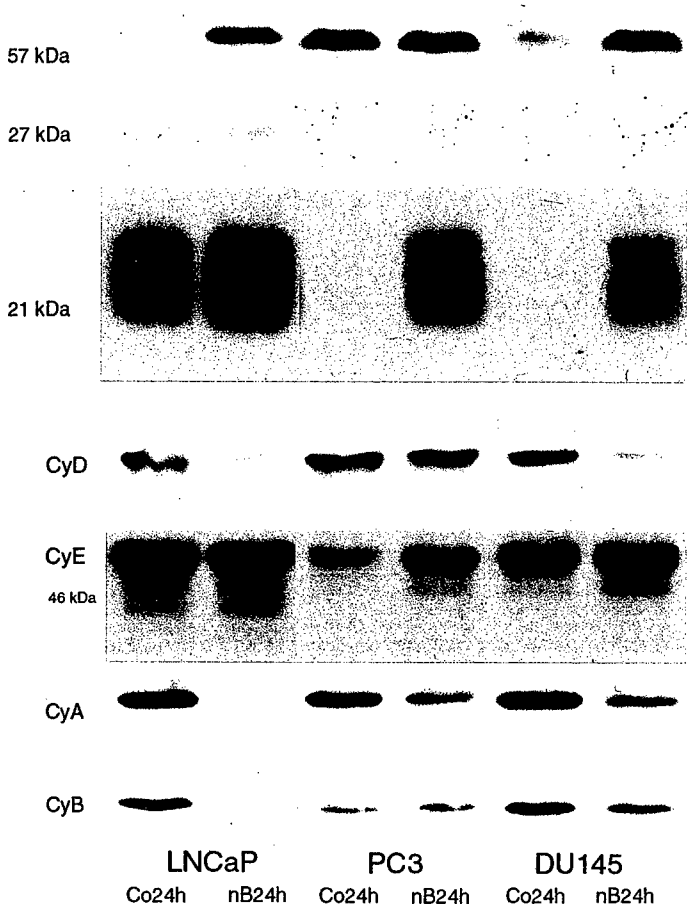


fig. 11a. CKIkip and cyclin expression in absence and presence of butyrate treatment

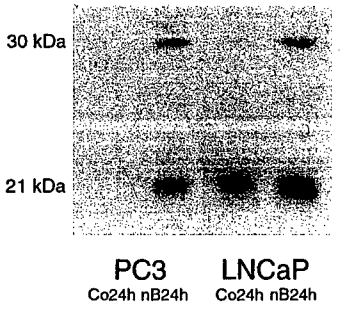


fig. 11b. Cyclin D3 and p21cip1 expression by LNCaP and PC3.
Cells were treated and analyzed using a single gel, with both antibodies, p21cip1 (PMNG 556431, MC) and cyclin D3 (SC6283, MC) used sequentially on a single blot. This ECLogram demonstrates that LNCaP cells express high basal levels of p21cip1 but have rather low basal expression of cyclin D3, while PC3 cells have low basal expression of each. Both PC3 and LNCaP appear to express maximal levels of cyclin D3 and p21cip1 after butyrate treatment (5 mM).

not resecting cancer-involved prostate when there is a likelihood of preexisting extraprostatic growth. Again, this was consistent with our findings in colorectal cancer. The prostate cancers that we obtained for the current study would under acceptable clinical practice have been no greater than T1B-2 (homologous to Dukes' stage A and B cancers). What is still unknown is the proportion of metastatic cancers that can yield autonomous growth and whether there is a class of such cancers that will require stromal cells or their products for continued viability or growth. We have been severely disappointed at our lack of success over three years of attempting to secure funding for such a study.

Attempts to develop a program aimed at culture of metastatic prostate cancer cell lines from bone marrow biopsy and continuous bone stromal cell support.

After >12 months, intently maintaining primary prostate cultures for a time sufficient to select for autonomous cells, we concluded that the exercise was too costly in time and money. In the end, we had seen no prostate cancer cell cultures progress beyond 3-4 passages. Furthermore, we had only limited success evaluating the HDI-response of these cultures or the highly similar cultures of normal prostate epithelia. The finding that normal prostate epithelium (NPE) and possible prostate cancer cells senesced at the same time as the endogenous stromal cells led us to attempt several strategies for coculture with exogenous stromal cells, attempting to extend the replicative period of NPE and cancer cells. At the same time, it occurred to us that the best source of tissue would be that involved of skeletal metastases, while normal bone stromal cells could be cultured to continually supply the nascent cell lines with 'feeder' cells. While trying to solicit our clinical oncologists to requisition and/or inform us of autopsies on prostate cancer patients, Marc Ernstoff, M.D., an enthusiastic, senior urologic oncologist from NCCC suggested that we might consider bone marrow biopsy tissue obtained as part of a designated clinical trial. We thus designed and obtained IRB approval for a pilot study aimed to determine the feasibility of cell culture methods for deriving continuous metastatic prostate cancer/bone stroma cocultures. This study was intended initially for laboratory research alone but was designed to demonstrate the value of patient tissue culture methods as an adjunct to clinical treatment trials. Laboratory-derived correlates of drug action could thus be obtained on the same tumor cells that were the target of drugs *in vivo*. Although the value for rapid diagnosis may be limited, patient cancer / bone stroma cocultures might provide results where investigations of small numbers of fresh, isolated cells had failed. While the work was considered rational and timely, we have found little support among study section members of research funding agencies, who were unenthusiastic, leaned toward mechanistically-directed competitor proposals, saw little value in the establishment of cell lines, or considered the endeavor to be 'descriptive'. Most frustrating, when our hypothesis that requirements for stroma would persist in cell lines from metastatic prostate cancers was presented, offering that product cell lines could be used for testing the hypothesis and subhypotheses addressing the nature of essential products of stroma, the reviewers judged the work to be lacking a hypothesis. We were however successful in obtaining a small grant for pilot laboratory studies and partial salary, from the Frank Stanley Beverige Foundation. Obviously, the resources provided by the DoD/CDMRP Prostate were in no small measure critical for our development to date of the stromal coculture methods.

We adopted and modified published methods for explant culture of cancellous bone [32], leading to secondary, replicate cultures of bone-derived stroma. Basically, cancellous bone was minced to fragments of 1 mm, agitated to remove red marrow and lipid, and cultured in a medium supplemented with serum, hydrocortisone and ascorbic acid 2-phosphate. After the fragments had attached to the culture plastic (one week), and the associated mesenchymal cells had begun to migrate onto the dish, the medium was exchanged on a biweekly basis. After approximately one month, the dense stromal matrix and associated cells were digested to set up replicate secondary cultures, that were then recultured until use as feeder support of fastidious prostate cancer cell lines (e.g., PC3 and LNCaP - figure 12a) and for

Figure 12. Effect of stromal coculture on colony forming efficiency and butyrate response.

Bone stromal cell cultures for feeder support of cell line CFE were secondary cultures of bone derived mesenchymal cells and associated matrix. Femur heads were grossly sectioned and mounted for slicing of trabecular bone. Minced spongy bone was freed of hematopoietic cells and fat by shaking in saline, and placed in T75 tissue culture flasks with 'Skelgro' culture medium (DMEM supplemented with 8% fetal bovine serum, ascorbic acid, hydrocortisone, sodium pyruvate, non-essential amino acids and gentamicin). Bone chips were allowed to adhere to culture dishes during incubation for 7-10 days. Cultures were thereafter refed semiweekly with Skelgro, leading to removal of residual hematopoietic and lymphoid cells. Cell migration from bone chips onto plastic was seen during the second or third week of culture, and matrix deposition was progressive thereafter, leading to increasing intercellular separation by matrix and mineralizing 'bone nodule' formation. Once cultures had become dense (approx. 4-6 weeks), cells are passaged using a sequential digestion with collagenase followed by trypsin-EDTA. The cell suspension was sieved through 40 μm Nitex mesh (removing residual or enlarged bone chips), washed and seeded into replicate culture dishes. These 'E1p1' cultures were ready for studies of epithelial growth promotion after an additional 2-3 weeks of growth and differentiation.

Colony forming efficiency Fourteen day old E1p1 cultures of bone stroma were used to determine the effects of stroma on colony forming efficiency. All experiments, including synchronous control CFE studies, were carried out in Skelgro. Tumor cell lines were removed from flasks with trypsin-EDTA, washed, sieved to obtain a single cell suspension and counted. Cell seeding densities between 10^2 and 10^5 epithelial cells in dense cultures of marrow stromal cells yielded reproducible CFE values within 7-21 days. In assays using butyrate, epithelial cells were plated 24-48 hrs before butyrate and/or TPA was added. Following 48 hrs of treatment, cultures were washed briefly with warm Skelgro, retaining non-adherent cells by centrifugation of spent medium and wash fluid, and cultures refed with fresh media. When colonies of greater than 50 cells were frequent, spent culture medium was removed and the plates allowed to air dry and then fixed with cold methanol. Tumor cells were stained with an anti-cytokeratin 18 antibody followed by avidin-biotin-peroxidase complex and diaminobenzidine (Vectastain). Colonies ($\geq$ 30 cells, stained brown by peroxidase/benzidine) were counted using a microprojector with a 17X objective.

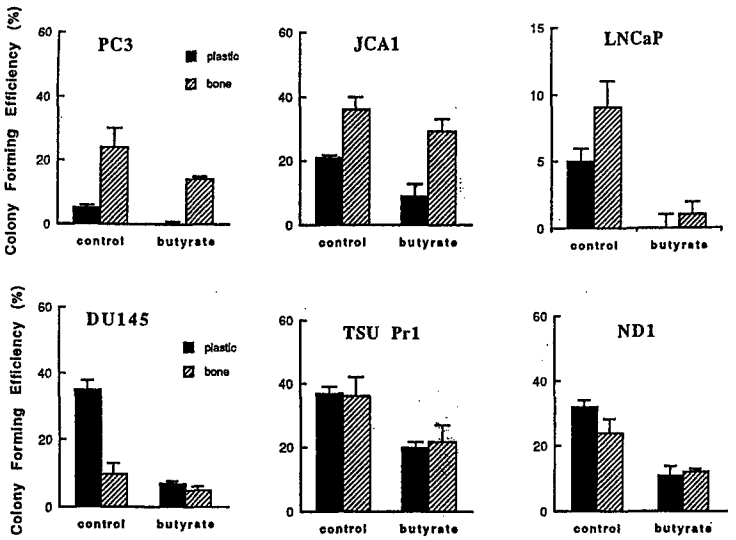


fig. 12a. Effect of butyrate and/or bone stroma on CFE
Single cell suspensions derived from each of the cell lines were plated on culture plastic or bone stromal cells/matrix (secondary cultures), and after 2 days treated with 5 mM butyrate or 1/3 volume fresh medium (control) for an additional 48 hr. After 15-21 days, colonies were fixed in methanol and stained with antibody to cytokeratin 8 with a peroxidase-conjugated secondary antibody and benzidine chromogen. The data represent average number of epithelial colonies divided by the number of cells plated X 100%, +/- standard deviation.

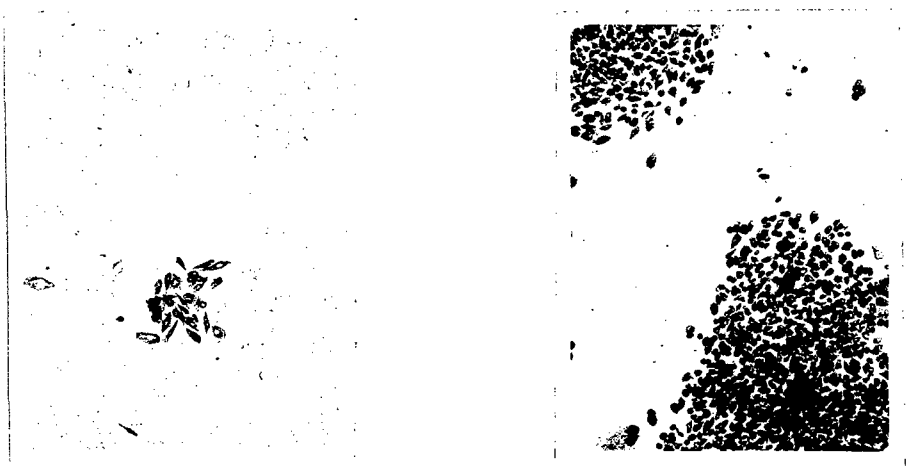


fig. 12b. Representative colonies of PC3 plated on culture plastic or bone stroma, grown for 21 days, fixed in methanol, stained with antibody to cytokeratin 8 (CAM 5.2, Becton Dickinson Inc.) and visualized with a peroxidase/benzidine ABC method (Vectastain™). The picture on the left is of a single colony of PC3 cells on plastic. In the picture on the right, two representative colonies are partially shown, with the bone stromal cells in the background poorly visualized in this non-counterstained dish.

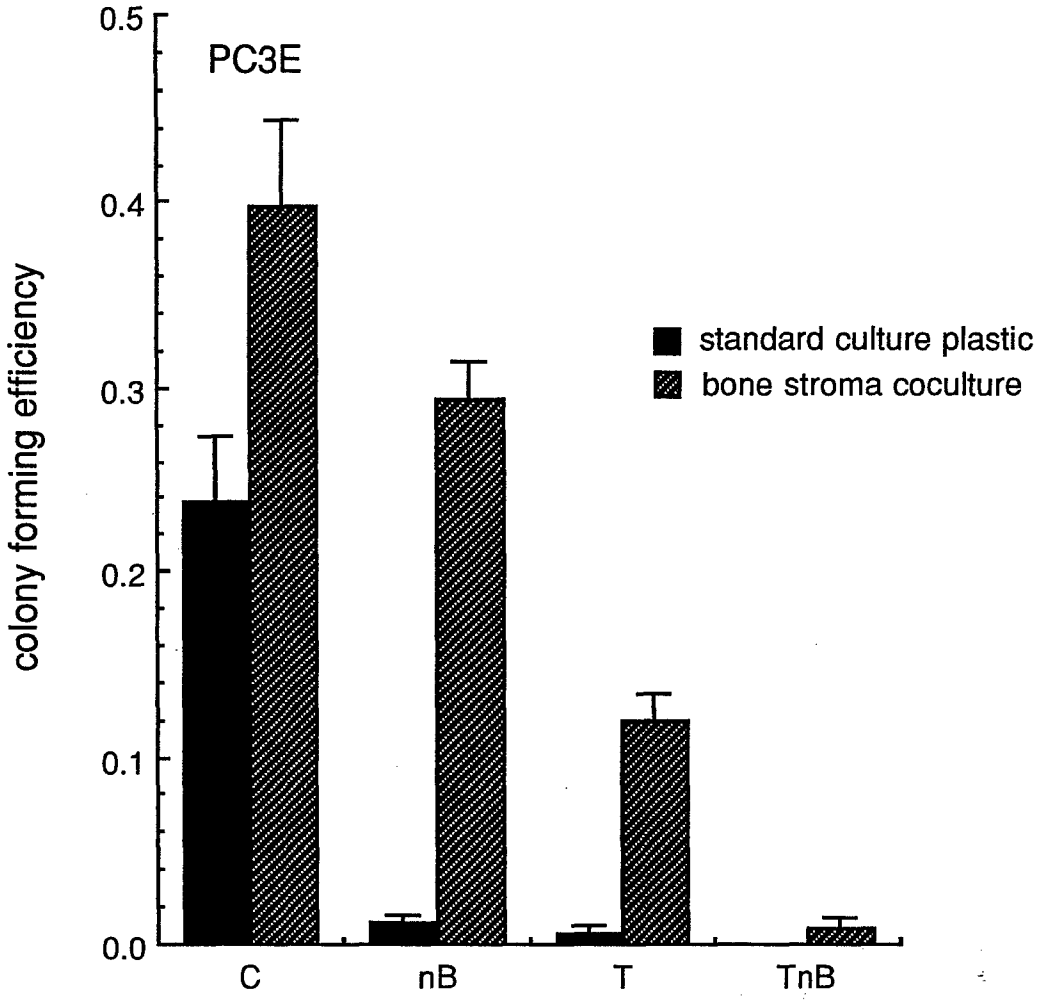
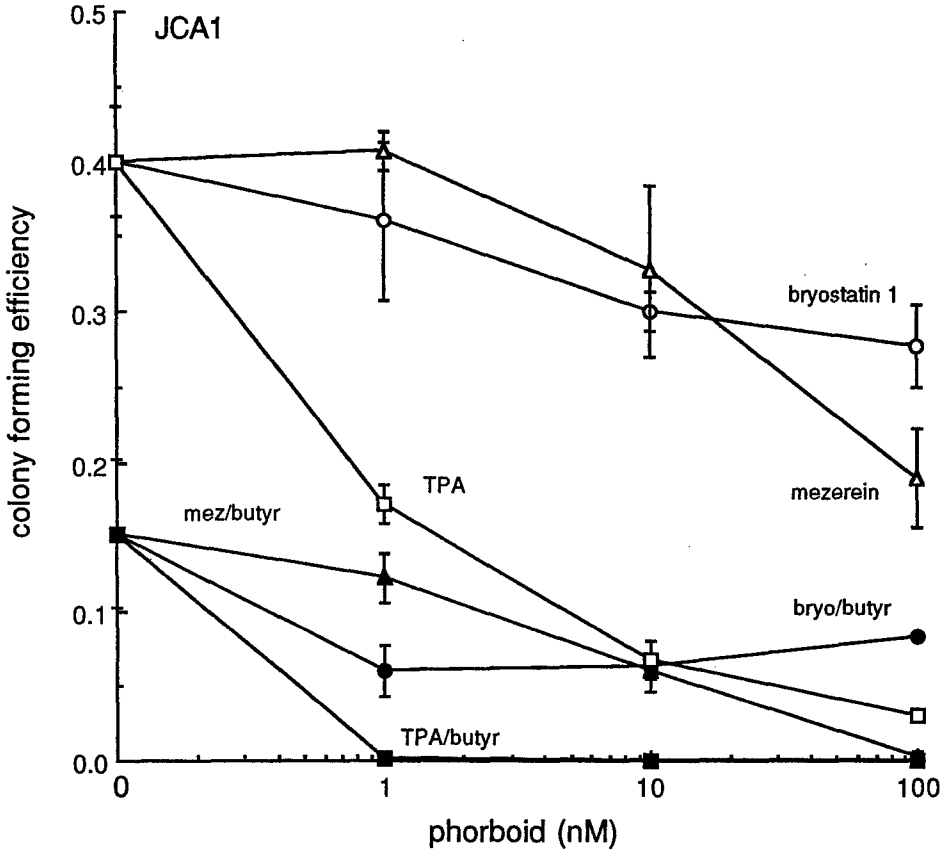


fig. 12c. Effect of butyrate +/- TPA on CFE of PC3E cells on plastic or bone stroma.



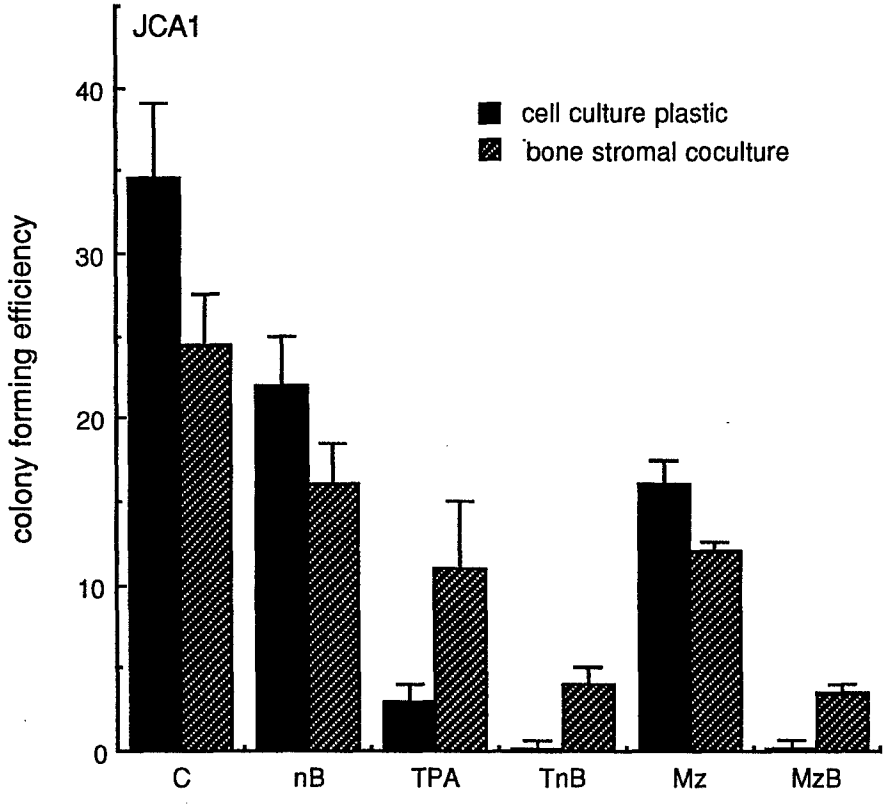


fig. 12d. Effect of TPA, mezerein or bryostatin 1 +/- butyrate on CFE of JCA1.

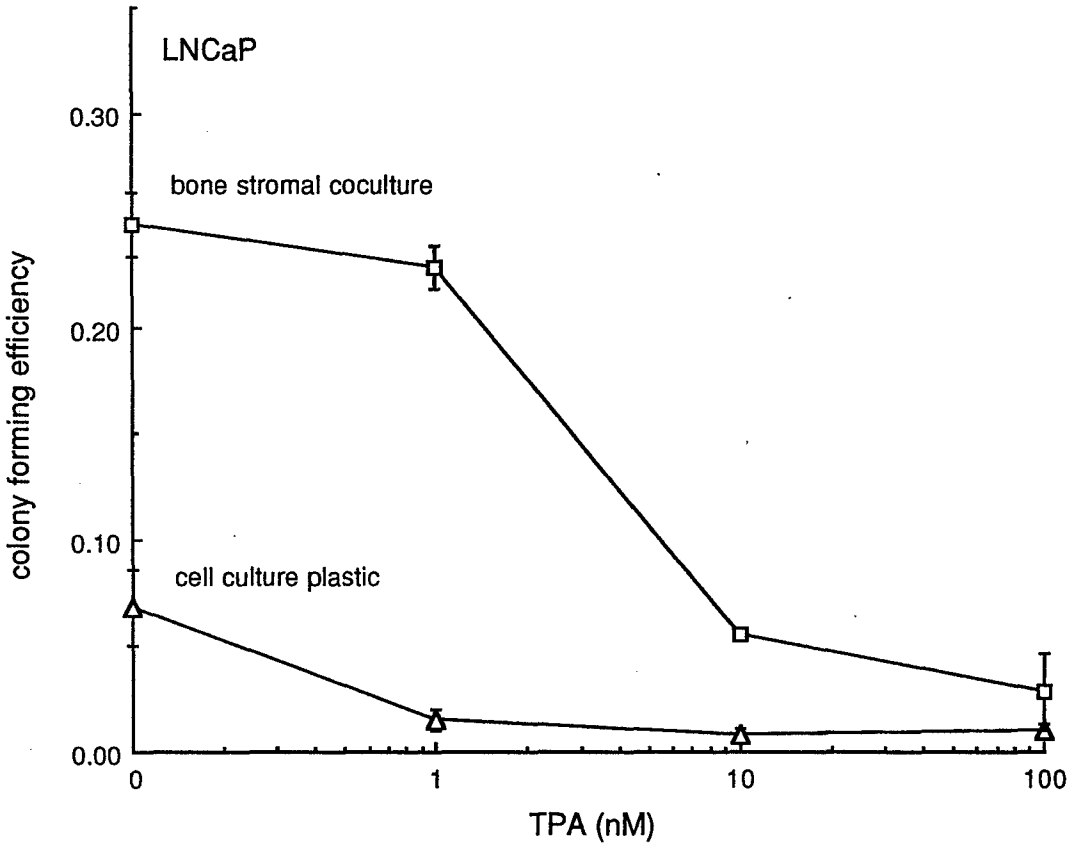


fig. 12e. Effect of bone stroma on TPA response by LNCaP.

characterizing osteogenic differentiation (VonKossa stain for mineral, and osteocalcin or alkaline phosphatase as osteoblast products). The preliminary data obtained with these cocultures included demonstrations of the competence of this format for isolation of minimal numbers of cancer cells as might be obtained from patient marrow biopsies, and for discrimination of epithelial cell colonies from stroma. Immunohistochemical staining methods, using antibodies to cytokeratins and EpCAM, have proven especially versatile for counting cancer cell colonies on a background of stromal cells and matrix. For the purposes of this report, such cultures were used to test for differences in survival of prostate cancer cell lines treated with butyrate or SBHA, and with TPA or mezerein. It is clear that stromal coculture provides a nurturing activity beyond secretion of soluble growth factors. But the practical benefit of improving colony forming efficiency and size uniformity cannot be dismissed, and such an approach may allow measurement of drug response and survival of cancer cells growing in a bone-like environment.

Effect of bone stroma coculture on basal CFE, apparent HDI tolerance and funding success

We have found that the colony forming efficiency of LNCaP and PC3 prostate cancer cells is 2-5X greater, respectively, when cocultured on bone stroma than when cultured in bone growth medium (figure 12a). Stromal cell coculture also dramatically protects PC3, and possibly DU145, JCA1 and LNCaP, from HDI-induced depression of CFE (figure 12a). Using the PC3E strain (a CFE-enhanced variant), stromal enhancement of CFE after butyrate treatment was seen to be independent of CFE enhancement by stroma, which is minimal in this subline. The PC3E subline emerged spontaneously during labored growth of PC3 cells under conventional culture conditions. The PC3 cell line is notorious for delay in reattaining logarithmic growth rates after passage, presumably due to the time needed for autocrine growth factors to reach sufficient levels. This burden may have been lifted somehow in the PC3E variant. However, the spontaneous transformation leading to growth enhancement for PC3E did little to increase tolerance to butyrate treatment (figure 12b)..

The mechanisms by which bone stroma coculture increases the CFE of cells like PC3 remain uncertain. Mechanisms for the protection of LNCaP by stroma, TSUPr1 by density or ascorbate/hydrocortisone, or PC3 by stroma are presumably distinguishable, and certainly remain to be elucidated. Interestingly, the trophic action of bone stromal cultures on the CFE of several lines was found to be partially transferable with spent 'conditioned' medium. However, on more careful examination we found that both the hydrocortisone and the ascorbic acid 2-phosphate supplements of the bone growth medium enhanced PC3 and LNCaP cell yields, and improved CFE after butyrate treatment (table 1). Similarly, CFE, colony cell numbers and HDI-protective activities are considerably greater if these cells are cultured in intimate contact with normal bone stroma than when growing in Transwell™ perfusion chambers suspended above bone stromal cells. Insights into the active constituents, or better, a cell-free preparation that recapitulates bone stroma would aid us in our quest for funding. As with many investigators, we found that support by stroma was recapitulated to only a limited degree by conditioned medium or by alkali/detergent-isolated extracellular matrix. Support of LNCaP cell CFE by stromal cell coculture was partially heparin-inhibitable, presumptive evidence of heparan sulfate proteoglycan-mediated and matrix-associated activities. These lines of investigation obviously require not only more work, but an innovative strategy to discriminate the effects of stromal matrix from direct stromal cell contact, as well as a means to harvest cancer cells in isolation from stroma. To this end, we hope to test a novel approach whereby epithelial cells will be separated from stroma using a microporous polycarbonate filter impregnated with collagen gel and gasketed into place over a precultured bone stromal cell mat (or a 250 μm cancellous bone slice). PC3 or LNCaP cells will be grown on the collagen investing this filter, and later removed by gentle scraping or collagenase digestion. Cyclin and CKIkip protein expression will be measured as a response to butyrate treatment or basal growth conditions. But for the absence of

funds and inescapable despair we would test the hypothesis that stromal matrix increases PC3 CFE through normalization of p57kip2 protein expression.

We have found that DU145, a prostate cancer line derived from a brain metastasis, responds in a distinctly different manner to bone stroma. Its CFEbone reflects up to 80% inhibition relative to CFEplastic, independent of the ability of the same donor bone cultures to enhance CFE of PC3 and LNCaP. Interestingly, CFEbone for DU145 treated with butyrate is nearly equivalent to the CFEplastic for butyrate-treated DU145, i.e., butyrate has subadditive inhibitory effects on DU145 considering the reduced CFE of DU145 cells cocultured with bone stroma. We offer two possibilities for this: 1) The same 70-80% of the population that dies after HDI treatment is also hypersensitive to a product of bone stroma, eg., TGFbeta, or 2) the cell population that adapts to plating on stroma is (also) protected against HDI-induced cell death. We would test the sensitivity of DU145 and ND1 (stroma-tolerant DU145 derivative) to TGFbeta, and then test for activity of receptor blocking antibodies or soluble receptor constructs in selectively improving the CFE of DU145 on bone stroma.

5. Conclusions

Conclusions we have drawn from this study include both those related to the appropriateness of culture methods for comparing HDI-response, and to potential mechanisms of tolerance of HDI-induced growth arrest.

The specific conditions used to test for recovery or persistent inhibition after transient HDI-treatment had a dramatic effect on outcome. Colony forming efficiency determinations provided the best illustration. The TSUPr1 cell line, but not its G2 arrest-deficient relative JCA1, can appear fully susceptible to irreversible HDI-induced growth arrest or death in a CFE determination; the ND1 variant of DU145 can also appear uniformly susceptible. If however the CFE assay includes ascorbate and hydrocortisone, supplements used for osteoblast culture or coculture, or if the cells are tested in mass culture, then survival after HDI-treatment is considerably increased. Similarly, the PC3 cell line appears fully inhibited in CFE and in cell replication in mass culture when treated with HDI. When tested in coculture with bone stromal cells, the basal CFE of PC3 cells is increased about 5-fold, while the CFE after HDI treatment was enhanced over 100-fold. Considering the minimal evidence of cell death in butyrate-treated PC3 cells, these cells appear to suffer an unusual increase in growth requirements superimposed on an already fastidious phenotype. The CFE-enhanced variant of PC3 that emerged during continued passage, PC3E, retained susceptibility to HDI-induced CFE inhibition and to stroma assisted resistance to butyrate-induced CFE depression. The phenotype of this PC3E variant suggests that stroma-supported HDI tolerance is not simply due to CFE enhancing effects of stroma, but rather an additional level of support that may be homologous to the effects of ascorbate and hydrocortisone on TSUPr1 and ND1.

The molecular mechanisms of HDI tolerance are currently unknown, but may plausibly involve CKIkip cell cycle regulatory proteins and their binding partners. Not only does the relative abundance of p21cip1 correlate with surviving fraction, but an even stronger relationship holds for the ratio of p21cip1 to cyclin E, a potential indicator of the relative level of free p21cip1. While surviving fraction falls at a similar rate in JCA1 and TSUPr1 cultures upon HDI treatment, the later cell line manifests HDI-induced G2 arrest that is not seen in JCA1. Instead, JCA1 arrests with a G1 complement of DNA. However, tolerance of JCA1 to HDI treatment is transitory, abating in conjunction with a resumption of DNA replication by JCA1 cells. For cell lines such as LNCaP, at least when growing on standard (suboptimal) culture substrates, p21cip1 is expressed at a moderate basal level and appears to be refractory to further induction. Again, the decline in relative abundance of cyclin-free p21cip1during HDI treatment may correlate with apoptosis. Considering that cyclins D3 and E continue to be expressed at a persistently

high level in HDI-treated LNCaP, it is possible that the point of intolerability occurs when HDI-treated cells no longer have sufficient p21cip1 in excess of cyclin/CDK complexes. Refractoriness to HDI-induced p57kip2 expression by PC3 and HDI-intolerance may also be related to growth under suboptimal conditions, and may be tested in conjunction with efforts to improve the microenvironment for culture of fastidious prostate cancer cell lines. A model system attending to requirements for prostate cancer cell line survival would be prime for identifying agents that could interfere with stroma-supplied protective factors. Such blocking agents might synergize with HDI agents in treatment of bone metastases of prostate cancer.

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